Quantification of Tyrosine Hydroxylase and ErbB4 in the Locus Coeruleus of Mood Disorder Patients Using a Multispectral Method to Prevent Interference with Immunocytochemical Signals by Neuromelanin.

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Quantification of tyrosine hydroxylase and ErbB4 in the locus coeruleus of mood disorder patients: using a multispectral camera to prevent the interference of neuromelanin with the immunocytochemical signals

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Abstract

The locus coeruleus (LC) was studied ~~is involved in stress regulation and mood disorders. It remains unclear whether~~ in major depressive disorder (MDD) and bipolar disorder (BD)~~ show similar LC activity~~. A major problem of immunocytochemical studies in the human LC is the interference of the omnipresent natural brown-pigmented neuromelanin with the staining of the immunocytochemical end product. Here we used a multispectral method~~camera~~ to untangle the two colors: ~~the pure spectrum for the~~ blue immunocytochemical ~~staining was obtained in a brain area without neuromelanin while the pure spectrum of~~ brown for neuromelanin. ~~was based upon a 'blank' LC slide, for which the primary antibody was replaced by buffer.~~ We observed significantly increased tyrosine hydroxylase (TH) in the LC of MDD (P=0.008) – thus validating the method- but not in BD (P=0.560) patients, and we did not find significant changes in the receptor tyrosine-protein kinase ErbB4 in the LC either in MDD or BD patients. We observed clear co-localization of ErbB4~~-ir~~, TH~~-ir~~ and neuromelanin in the LC neurons. The different stress-related molecular changes in the LC may contribute to the different clinical symptoms in MDD and BD.

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Introduction

Major depressive disorder (MDD) and bipolar disorder (BD) are the two major subtypes of mood disorders, which affect over 17% of the world's population and cause a heavy medical and social burden to individual, family and society [1]. Functional changes in emotion- and stress-related neuronal-networks are proposed as the core pathogenesis of these mood disorders – with clear individual differences, which lead to a greater vulnerability to the stress response [2]. The locus coeruleus (LC) and the hypothalamo-pituitary-adrenal (HPA)-axis are two important hubs in this network that interact closely [2-6].

The LC is located in the brain stem, its neurons contain neuromelanin and it is the main source of norepinephrine (NE) in the brain. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for NE production [7] and is regarded as a good biosynthetic index of LC activity [8]. The LC receives corticotropin-releasing hormone (CRH) projections from the hypothalamus, while NE may stimulate HPA activity [3]. In rats, chronic stress was found to increase LC-TH expression [9, 10] and LC electrostimulation led to increased plasma adrenocorticotropic hormone levels, indicating CRH activation [11]. In addition, increased TH levels were found in the rostral, middle and caudal parts of the LC in MDD patients [6], together with elevated NE in the cerebrospinal fluid (CSF) and

increased plasma cortisol, indicating activation of both, the LC and the HPA-axis. Moreover, elevated CSF NE and plasma cortisol were observed in melancholic depression patients [3]. Furthermore, higher LC activity was found in suicide depression patients, especially in those with violent suicidal behaviors [12, 13]. There are only a few studies that address LC activity in BD patients. Lower LC TH-immunoreactivity (-ir) was observed in suicide BD patients compared with normal controls [8], but no comparison was made between BD patients that did not commit suicide and controls. It remains therefore, unclear whether BD and MDD patients show different changes in LC activity.

It is also of interest to note that, recently, the role of ErbB4 in the LC in schizophrenia and BD has drawn a lot of attention [14-16]. ErbB4, as a receptor tyrosine kinase, is a member of the epidermal growth factor receptor–subfamily, which is essential for many neuro developmental processes, including synapse formation, neuronal differentiation, axon navigation and myelination [17]. ErbB4 is phosphorylated after binding to its ligand neuregulin (NRG)-1, followed by dimerization with ErbB2, leading to subsequent phosphorylation and activation of signaling pathways such as Akt and extracellular-regulated kinase (ERK) signaling pathways [1, 18]. ErbB4-mRNA was found to be widely expressed in NE neurons in LC in rats, which suggested a possible role of ErbB4 in the regulation of the LC-NE system [19]. In addition, a SNP study in Dublin showed that a polymorphism of ErbB4 was significantly related to schizophrenia [20]. Increased ErbB4-mRNA levels were found in the dorsolateral prefrontal cortex of schizophrenia patients compared with control subjects [21]. Since BD and schizophrenia share a similar

genetic background and similar clinical symptoms [22, 23], ErbB4-NRG1 expression was proposed to play a role in BD. In rats, it was observed that down-regulating ErbB4-NRG1 in hippocampus and prefrontal cortex was related to decreased glutamate decarboxylase (GAD)-67 and GABA levels in the same brain regions [1]. Recently, it was found that LC-ErbB4$^{-/-}$ mice exhibit certain mania-like behaviors including increased movement speed and distance in open field test (SX Cao, data unpublished).

It was~~is~~ thus considered to be important to determine quantitatively the changes of TH and ErbB4 in the LC of MDD and BD patients. However, there is an inherent problem of immunocytochemical studies of the human LC, which is the interference of the immunocytochemical staining products with the natural brown-pigmented neuromelanin that is present in the far majority of the large LC neurons. This pigment can even be useful as a marker when the number of immunoreactive LC neurons is counted, and quantification of the amount of immunocytochemical product per cell is not essential (Bouman et al., 1999; Gos et al, 2008). An alternative method is to extract proteins from LC tissue punches and to determine their amount (M. Zhu et al., 2000), but this may cause lost~~s~~ of the LC morphological characteristics and make impossible double labelling of the protein in LC neurons. It should be noted that fluorescence is also not be applied to the human LC for quantitative immunocytochemical research, due to the strong autofluorescence of human brain tissue.

Therefore, we used in the present study ~~we used~~ a multispectral camera to untangle the two colors: the genuine spectrum for the blue immunocytochemical staining was

obtained in a brain area without neuromelanin, while the genuine spectrum of brown neuromelanin was based upon a 'blank' LC slide, for which the primary antibody was replaced by buffer in the staining procedure. As a first essential step for further functional studies on the neuromelanin-containing LC in different mood disorders, we determined by means of the multispectral method the expression of TH and ErbB4 expression in BD or MDD patients in comparison with their respective matched controls. In addition, we studied the co-localization of TH and ErbB4 in neuromelanin-containing neurons of the human LC.

Materials and methods

Subjects

Postmortem LC samples were obtained from the Netherlands Brain Bank (NBB), Amsterdam. All material was collected from donors from whom written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB. Diagnosis of MDD or BD at any time during life was made by qualified psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-III-R/DSM-IV criteria. The absence of neuro-pathological changes, both in the mood disorder groups and the control groups, was confirmed by systematic neuro-pathological investigation [24].

The LC were dissected at autopsy and fixed in 0.1M phosphate buffered 4% w/v

formaldehyde (pH 7.2) for 1-2 months. Two separate control groups of 11 largely overlapping patients (9 each) were matched to 9 BD and 9 MDD patients respectively. For P values of matching see **Table I** and **Table II**. Please note that there was a trend of ($p = 0.075$) lower CSF-pH in BD group (mean = 6.34) compared with that of its control group (mean = 6.63). Lower CSF-pH has been observed as an endophenotype of BD [25] and can thus not be considered as a confounder. In addition, there was a significant difference between the death of month between MDD group (mean = July±2 months) and its controls (mean = March±2 months, $p=0.01$, **Table I**), to which we paid special attention for analysis (see Results and Discussion).

The brainstem, containing the LC, was dehydrated in graded ethanol, embedded in paraffin and serially cut in 6 µm sections along the rostro-caudal axis on a Leitz microtome and stored at room temperature (RT).Every 50th section was mounted to locate the midlevel part of the LC by the presence of the largest amount of brown pigmented neurons in unstained paraffin sections. The sections around the midlevel of LC were stained for TH or ErbB4, respectively. Subsequently, adjacent sections were used for double-staining of TH and ErbB4.

Patients were characterized as belonging to the suicide group if they experienced suicide ideations (n = 2), had attempted suicide (n = 2) or had committed suicide (n = 1). There were 3 BD suicide patients and 2 MDD suicide patients. In addition, there were 2 legal euthanasia MDD patients and 1 control subject who died by voluntary starvation (**Table I** and **Table II**), for whom we did extra analysis.

Immunocytochemical staining for TH

The specificity of the monoclonal mouse-anti-TH (Millipore MAB318) has been extensively confirmed by Western blotting and immunohistochemistry [26, 27]. One midlevel LC section per patient was used for TH staining. In brief, the sections were deparaffinized and rehydrated using xylene and decreasing grades of ethanol. After rising in aqua dest (1x5 min), the sections were placed in 0.05 M Tris-HCl (pH 9) and microwave-treated for 10 min at maximum power (800W) for antigen retrieval. After cooling down to RT, the sections were rinsed in TBS (pH 7.6) and incubated overnight at 4˚C with monoclonal mouse anti-TH at a 1:8000 dilution in supermix (0.25% gelatin and 0.5 ml Triton X-100 in 100 ml TBS, pH 7.6). On the second day, the sections were rinsed in TBS and incubated for 90 min with donkey-anti-mouse IgG conjugated to alkaline phosphatase (AP) (cat. no. 715-056-150, Jackson ImmunoResearch), 1:200 diluted in supermix at RT. After washing in TBS and pre-incubation in buffer (100 mM NaCl, 5 mM MgCl 2, 100 mM Tris-HCl pH 9.5), the LC was visualized with color solution containing 0.34 mg/ml nitro-blue tetrazolium chloride (NBT), 0.175 mg/ml 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) and 0.24 mg/ml Levamisole (L-9756, Sigma-Aldrich) in Buffer 2. Color development took place under dark conditions at RT for 30 min. Reactions were stopped by rinses in aqua dest (2x5 min) followed by methanol (1x5 min) to remove the brown discoloration, and in water (2x5 min) before coverslipping the sections.

Immunocytochemical staining for ErbB4

The immunohistochemical protocol for ErbB4 was largely similar to that of the TH staining, except that after rehydration, sections were boiled in 0.01M Citrate Buffer (pH 6.0). After a 1 hr wash in TBS 5% milk incubation to reduce the background, sections were incubated with a monoclonal rabbit anti-ErbB4 antibody (Abcam ab32375, lot number: GR3174336-3), at a 1:50 dilution. The specificity of this antibody has been proved by negative staining in LC-ErbB4$^{-/-}$ mice (SX Cao, unpublished data). Detection was performed by anti-rabbit IgG conjugated to Alkaline Phosphatase (AP) (cat. no. 711-055-152, Jackson ImmunoResearch, 1:1 in glycerol). Time needed for color development was 90 min. 2 sections per patient were used, and their average value was analyzed.

Double-staining of TH and ErbB4 in human LC

For the immunocytochemical study of the co-localization of TH and ErbB4, the same antibodies were used as in single staining. Double staining of TH and ErbB4 was performed on the section around midlevel of LC for each subject. The sections were deparafinizated and rehydrated in graded ethanols, and then placed in a microwave oven in 0.05 M Tris-HCl (pH 9) buffer for 10min. Sections were then incubated overnight in a mixture of Rabbit-anti-ErbB4 (dilution 1:15) and mouse-anti-TH (1:8000). The next day the sections were rinsed in TBS for 3x10min and subsequently incubated with anti-rabbit IgG conjugated to alkaline phosphatase (AP) (1:200) at RT for 1h. The visualization was obtained in blue for ErbB4 using Fast Blue kit (cat. No. SK-5300, Vector Laboratories) for 50 min. Sections were rinsed for 3 x 5min and then incubated

with biotinylated anti-mouse (1:200) at RT for 1h, then incubated with avidin-biotin complexes (ABC, 1:800) at RT for 1h. Visualization was obtained in red for TH using 3-amino-9-ethylcarbazole (AEC) red kit (cat. No. SK-4200, Vector Laboratories) for 50min.

Image analysis

A stack of images of each section was collected at different wavelengths ranging from 520 to700 nm in 20 nm steps on an Axioskop (Zeiss) microscope equipped with 20x (for TH) or 10x (for ErbB4) objective and a Nuance FX multispectral camera (Perkin Elmer). The pure spectra of the NBT/BCIP staining (blue) was based upon the ErbB4 staining of human hippocampus while the pure spectra of brown neuromelanin was based upon a 'blank' LC slide, for which the primary antibody was replaced by sumi in the staining process. This allowed to untangle these two colors (see below) [28] with the Nuance software (Version 3.0.1.2, Cambridge Research Instrumentation; Woburn, MA, USA; **Fig 1**).

When there was negligible or no bleed-through of neuromelanin into the blue spectrum, the Nuance software was used to analyze the blue spectrum. Because of the large inter-patient variance in background intensity, the mask threshold had to be set for each patient individually. Immunocytochemical signal was quantified by determining the optical density (OD) in an outline of the LC following thresholding: only signal above 2.5x (TH) or 2x (ErbB4) background density were masked and measured. Staining was assessed for its OD, and then multiplied by the area of the mask

to get the integrated optical density (IOD), which was subsequently corrected by the total area of LC in the same section to calculate the corrected IOD. Images to outline total area were obtained with the Axioskop 9811 microscope (20x objective for TH, 10x objective for ErbB4) with a Sony 77xc camera to tile multiple pictures into one large snapshot. Manual corrections of artifacts were kept to the absolute minimum. For TH, image analysis was largely similar to that of the ErbB4 methods. However, background intensity was lower and more consistent, and consequently all subjects were analyzed with the same threshold (2.5x average-background).

For the analysis of TH-ErbB4 double staining, Nuance 3.0.1.2 software was used to build up spectral libraries of the chromogens [28]. In brief, using the 40x objective, a single LC TH-stained or LC 'blank' slide, or hippocampal ErbB4-stained slide were scanned with the aim of defining the respective spectral curves, which were then used to distinguish the three chromogens (red, blue and brown) in the double-stained sections for signal quantification. To define the specific signal during masking, the OD threshold was set at 4 times the background for the red and blue chromogen. Subsequently, the co-localization of TH (red) neurons and ErbB4 (blue) was analyzed.

Statistics

All data were analyzed with IBM SPSS Statistics version 23. Data was first checked for normality with a Shapiro-Wilk test of normality and for equality of variance with a Levene's test. Since most data was normally distributed, differences in corrected IOD values between 2 groups were tested with Student-T test. In addition, the analysis of

covariance was applied to analyze the effect of CSF pH on TH-ir and ErBB4-ir expression. Pearson test was applied to analyze correlation. Since there was large overlap between the two control groups, Bonferroni correction was applied after t-tests. All tests were 2-tailed and $p \leq .025$ was considered significant.

Results

TH-ir increased in the LC of MDD but not of BD patients

TH-ir was present in the cytoplasm and fibers of neurons containing neuromelanin, both in controls and mood disorder patients. The distribution and the appearance of the TH-ir neurons in mood disorder patients were similar to those of the control cases, except that the intensity of the staining seemed to be higher in some of the MDD patients (**Fig 2A-D**). The corrected IODs of LC TH-ir showed a highly significant increase in the MDD group compared with its controls (p=0.008), while LC TH-ir in the BD group was not significantly different from its controls in this respect (p=0.560). The TH-ir of the 3 BD patients who had attempted suicide or had had suicide ideations (mean=0.013, range 0.002-0.033) was within the range of TH-ir of the other BD patients who had not attempted suicide and had not had ideation (n=6, mean=0.016, range 0.003-0.037). In addition, the TH-ir of the 2 MDD patients who had done suicide attempt or had had suicide ideation (mean=0.024, range 0.019-0.028) was within the range of TH-ir of the other MDD patients who had not attempted suicide and had not

had ideation (n=7, mean=0.029, range 0.011-0.058). When the 2 MDD patients who died of euthanasia were included in the suicide group, the TH-ir levels (mean=0.030, n=4, range 0.019-0.045) were not significantly different from the other MDD patients as well (mean=0.026, n=5, range 0.011-0.058; p=0.599, **Fig 2E**). In addition, the TH-ir level (0.013) of the control subject who died of refusing food and water was within the range of TH-ir levels of the other controls (mean=0.011, n=10, range 0.002-0.024, **Fig 2E**). Moreover, there were no significant differences of TH-ir levels between the subjects who died during June-August compared with those died in other months (for selection of these months see Discussion), either in the MDD group or in its control group (**Fig 2F**). Furthermore, there was no significant correlation between CSF pH and TH-ir, either in BD group (r=0.514, p=0.238) or in its control group (r=0.457, p=0.216). Lastly, with CSF pH as a covariant, there was no effect of BD group on the level of TH-ir ($F(1,16)=0.510$，$p=0.510$), although there was a trend of lower CSF pH effect on TH-ir level ($F(1,16)=3.835$, $p=0.074$).

No significant changes in the LC ErbB4-ir in MDD or BD patients

ErbB4-ir was present in the cytoplasm of the LC neurons containing neuromelanin, both in controls and mood disorder patients (**Fig 3A**). The distribution and the appearance of the ErbB4-ir neurons in mood disorder patients was similar to that of the controls.

There was no significant difference in the corrected IODs of LC ErbB4-ir between the MDD group and its controls (p=0.761), or between the BD group and its controls

(p=0.289, **Fig 3B**). There was no significant correlation between CSF pH and LC-ErbB4-ir, either in the BD group (r=0.563, p=0.188) or in its control group (r=-0.110, p=0.779). In addition, there was no significant effect of BD (F(1,16)=0.880, p=0.367), or of CSF pH (F(1,16)=0.226, p=0.643) on the level of ErbB4-ir. And the interaction between BD group and CSF pH was not significant (F(1,16)=0.853, p=0.374), either.

No significant correlation was found between LC-ErbB4-ir and LC-TH-ir in the pooled controls (n=11, r=-0.192., p=0.572), in the BD patients (n=9, r=0.116, p=0.766) or in the MDD patients (n=9, r=-0. 217, p=0.575).

Co-localization of TH and ErbB4 in the LC

ErbB4-ir and TH-ir showed a clear co-localization in the LC of MDD, BD patients and controls (**Fig 4**).

Discussion

In the present study w~~W~~e validated our method of quantitative immunocytochemical analysis formalin-fixed paraffin-embedded postmortem human LC tissue ~~in the present study~~ by confirming that TH-ir was significantly elevated in the LC of MDD patients. This is in line with an earlier study finding (ref), which was performed by an independent technique, i. e. protein extraction from LC punches. A novel observation was that there was no increased TH-ir found in BD patients. This indicates a different

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state of LC activity between these two subtypes of mood disorders, which may contribute to the different clinical symptoms seen in these two disorders. It should, however, be noted, that none of the BD patients in this study had died in a manic stage. Thus, although it seems that LC-TH-ir change is not a trait marker of BD, further study to see whether LC-TH-ir changes in the manic phase of BD patients is warranted, if such material can be obtained. In addition, the method we used in the present study allowed us to show that ErbB4-ir and TH-ir were clearly co-localization in the neuromelanin-containing neurons of the LC in MDD, BD patients and in controls.

Baumann et al. (1999) found that the TH-ir level in LC in depressed suicide patients was higher than that in non-suicide depression patients [12]. Our data do not support these results, either in BD or MDD, since they were all in the same range as the depressed patients that did not commit suicide. However, it should be mentioned that at one hand Bauman et all did not express their data as corrected IOD, while at the other hand the very small number of suicide patients calls for confirmation. It should also be noted that some study found that people who died in summer period (June to August) showed higher density of TH-expressing neurons in the midbrain than those who died in winter (November to January) [29]. Although in the present study the average season (month) of death of MDD group was July while its control group was March, we did not observe significant difference of TH-ir levels between those who died during June to August and those who died in other months, either in the MDD group or in its control group (**Fig 2F**). It deserves to study the biological rhythms of TH-ir in the LC.

We did not observe significant changes in LC-ErbB4-ir either in MDD or in BD patients, compared with their respective controls, which implies that LC-ErbB4 might not show a major change in these mood disorders. Clear co-localization of ErbB4 and TH was observed in the human LC, which is in accordance with the findings in mice [19]. Chronic unpredictable mild stress rats, an animal model for depression, showed elevated phosphorylated-ErbB4 and NRG1 in the prefrontal cortex and decreased phosphorylated-ErbB4 and NRG1 in hippocampus [18]. Recently it was found that LC-ErbB4$^{-/-}$ mice exhibit certain mania-like behaviors (SX Cao, data unpublished), and polymorphism in ErbB4-NRG1 pathway are risk factors for schizophrenia [20]. However, to the best of our knowledge, we are the first to study the possible presence of alterations in LC ErbB4 in MDD and BD patients. We found that although ErbB4 is co-localized with TH in human LC neurons, suggesting a possible role of ErbB4 on LC activity, there was no correlation between the TH-ir and ErbB4-ir expression. In addition, we did not find any significant change in ErbB4-ir, either in MDD or in BD patients. Thus a similar change in the LC as observed in the ErbB4$^{-/-}$mouse that showed manic-like behavior (see Introduction) was not observed in the human LC-ErbB4 levels in mood disorders. However, it should be mentioned that the human LC is much larger than that of a mouse. The LC of both mouse and human has topographic organization, i.e. different parts of LC project to different brain regions, carrying on different functions. For instance, the dorsal part of LC cells project towards neocortex and hippocampus, the ventral part of LC project towards the spinal cord, basal ganglia, thalamus, hypothalamus and cerebellum [30, 31], the rostral part of LC neurons project to the

forebrain, and the caudal part of LC-NE neurons project to the spinal cord [31]. Since we used the midlevel LC sections in the present study, the ErbB4 expression might not reflect the ErbB4 expression in the whole LC but may be present solely in a subarea that is more related with BD symptoms. The presence of possible region-specific alterations in ErbB4-ir in LC still needs further study.

One of the inherent potential confounding factors in a postmortem study is medication use. Indeed, one animal study showed that the selective serotonin reuptake inhibitors (SSRIs), which were used by some MDD patients in the present study, could delay the TH-ir decrease in rat LC after chronic stress [32]. However, our study showed that the LC TH-ir levels of the MDD patients were increased, and LC TH-ir levels of those who used SSRI (n=2, mean=0.024, range 0.019-0.028) were in the range of those MDD patients who did not use SSRIs (n=7, mean=0.029, range 0.011-0.058). In addition, animal studies have shown that benzodiazepines, for example olanzapine, may elevate LC TH-ir [33, 34]. However, we do not think that our main conclusion is confounded by this medicine since the TH-ir levels in BD patients who had used benzodiazepines (n=4, mean=0.011, range 0.002-0.033) were not different from those of the other BD patients, who had not used such medicines (n=5, mean=0.018, range 0.003-0.037, p=0.286). In addition, the TH-ir levels of 2 MDD patients who had used benzodiazepines (mean=0.030, range 0.027-0.032) was not different from those of the other MDD patients who had not used it (n=7, mean=0.027, range 0.011-0.058). Lithium was commonly used in many patients of the present study (See **Table I**). However, it has been reported that lithium does not affect TH-mRNA levels in the

ventral tegmental area in rats [35]. Moreover, anti-psychotic medication was reported to decrease CSF-NE levels in BD patients in the manic stage, who then changed into depressive stage [36, 37]. In our study, the LC TH-ir in BD patients who used anti-psychotic medicine (n=5, mean=0.014, range 0.003-0.033) was not significantly different from the BD patients who had not used such medicines (n=4, mean=0.016, range 0.003-0.037, p=0.850). Furthermore, the LC TH-ir of MDD patients who had used anti-psychotic medicine (n=2, mean=0.012, range 0.011-0.012) were below the range of the ones who had not used it (n=7, mean=0.033, range 0.018-0.058), so if they had an effect, it has led to an underestimation of the increase in TH-ir. For ErbB4 such animal studies with medicines are not available.

Conclusion

The multispectral method allowed us to determine quantitatively immunocytochemical changes in the LC neuromelanin-containing neurons. In addition, we established with this method the presence of co-localization of TH and ErbB4 in these neurons of the human LC. The LC was found to be significantly activated in MDD but not in BD patients, which may contribute to the difference in clinical symptoms between MDD and BD. ErbB4 changes were not observed in the human LC, but material of patients that died in a manic state was not available.

Acknowledgements

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