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Up-regulation of neuronal calcium sensor-1 (NCS-1) in the prefrontal cortex of schizophrenic and bipolar patients

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The delineation of dopamine dysfunction in the mentally ill has been a long-standing quest of biological psychiatry. The present study focuses on a recently recognized group of dopamine receptor-interacting proteins as possible novel sites of dysfunction in schizophrenic and bipolar patients. We demonstrate that the dorsolateral prefrontal cortex in schizophrenia and bipolar cases from the Stanley Foundation Neuropathology Consortium display significantly elevated levels of the D2 dopamine receptor desensitization regulatory protein, neuronal calcium sensor-1. These levels of neuronal calcium sensor-1 were not influenced by age, gender, hemisphere, cause of death, postmortem period, alcohol consumption, or antipsychotic and mood stabilizing medications. The present study supports the hypothesis that schizophrenia and bipolar disorder may be associated with abnormalities in dopamine receptor-interacting proteins.

Abnormalities in the brain's dopamine system have been postulated for both schizophrenia (SCHIZ) and bipolar disorder (BPD) (1–4). However, in both cases, the exact sites of alterations in this system have remained elusive. The traditional focus on the regulation of dopamine and its receptors has been giving way in recent years to an increasing interest in intracellular signaling cascades and molecules (5–7). In particular, recent discoveries of multiple dopamine receptor-interacting proteins (DRIPs) and evidence of their role in modifying and expanding the functionality of these receptors has led us to hypothesize that abnormalities in the dopamine systems of SCHZ and BPD patients might lie in altered levels of certain DRIPs in specific areas of the brain (8–11). By using tissue from SCHIZ, BPD, major depression (MD), and normal control (NC) individuals provided to us by the Stanley Foundation Neuropathology Consortium, we have previously demonstrated that the dorsolateral prefrontal cortex (DLPFC) of SCHIZ patients expresses nearly twice the normal levels of the D1 DRIP protein, calcyon (12).

The present paper describes examinations of another DRIP, neuronal calcium sensor 1 (NCS-1), in the same tissue samples. NCS-1 belongs to the recoverin subfamily of EF-hand Ca^{2+}-binding proteins (13) and is present in neuronal cells throughout the brain (14–17). It has been shown that this protein can form complexes with G-protein-coupled receptor kinase-2 (GRK2) and D2 dopamine receptor (DR2), and, in doing this, prevents GRK2-mediated desensitization of activated DR2 (11). The regulation of DR desensitization is not the only reported activity of this protein. Among the major actions of NCS-1 and its *Drosophilae* orthologue, frequenin, are regulation of exocytosis of secretory and neurotransmitter substances (18–22), control of trafficking of cellular proteins (23, 24), and modulation of the activity of K^+ and Ca^{2+} ionic channels (25–27). In addition, up-regulation of the NCS-1 gene has been observed in the rat dentate gyrus after long-term potentiation, suggesting that this protein may be involved in activity-dependent neuronal plasticity (28). Here, we report that the levels of NCS-1 are significantly elevated in the DLPFC of SCHIZ and BPD patients. This finding supports our hypothesis that SCHIZ and BPD may be associated with altered levels of brain DRIPs.

Materials and Methods

The samples of human DLPFC (area 46 of Brodmann; ref. 29) of NC, schizophrenic, BPD and MD cases ($n = 15$ per group) were obtained from the Stanley Foundation Neuropathology Consortium. The DLPFC was chosen for analysis as displaying one of the highest NCS-1 expression levels in the brain (17). This region has also been identified as a site of dysfunction in both SCHIZ and affective disorders (reviewed in refs. 30 and 31). The demographic, clinical, and storage characteristics for cases constituting the Consortium have been published (32, 33). The diagnostic groups were matched according to age, race, gender, mean postmortem interval, pH, and hemisphere. Each sample was received as a set of six consecutive 40-μm-thick cryostat-cut frozen sections, which were maintained frozen after the sectioning. The cortical tissue from five sections per sample was cut out and homogenized for use in slot blot analysis. The sixth section from each sample was stained with cresyl violet and used for estimation of the proportion of neuronal cells within the cortex. All of the analyses in this study were performed in a blind manner, with the diagnosis of the cases being revealed to the investigators only after all of the data were collected.

Samples of monkey DLPFC (area 46 of Brodmann; ref. 29) were obtained from five drug-naïve control and five haloperidol-treated rhesus monkeys. The latter animals received 0.175 mg/kg haloperidol orally twice a day for a period of 6–8 months. Previously we demonstrated that such treatment results in up-regulation of DR2s and down-regulation of DR1s in DLPFC (34). A portion of the dissected tissue was homogenized as described above for the human samples. The other part was frozen, sectioned on a cryostat into 40-μm-thick sections, and processed for cresyl violet staining.

Levels of NCS-1 in the sample homogenates were evaluated by using slot blots on NitroPure membranes (Osmonics, Minnetonka, MN) prepared with Bio-Dot SF Microfiltration Apparatus (Bio-Rad) as described (12, 35). We generated a total of 15 membranes from the human material, each containing blots of 12 samples in triplicates. Therefore, each of the 60 human samples used in this study was blotted on three different membranes. In addition, we produced three membranes, each of containing triplicate slots of all 10 nonhuman primate samples examined in this study. All membranes also included six slots of

Abbreviations: SCHIZ, schizophrenia; BPD, bipolar disorder; DRIP, dopamine receptor-interacting protein; MD, major depression; NC, normal control; DLPFC, dorsolateral prefrontal cortex; NCS-1, neuronal calcium sensor protein 1; DR2, dopamine receptor 2.

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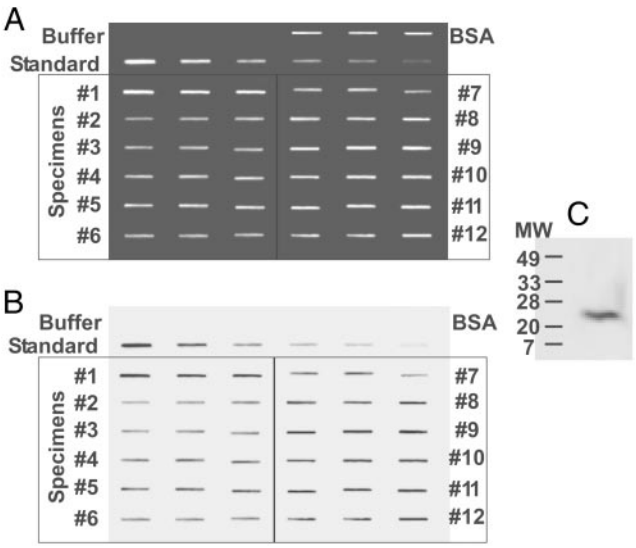

Fig. 1. Representative examples of the images of slot blots and Western blots generated in this study. (*A*) Typical fluorescent image of a membrane with slot blots stained for the total protein. (*B*) Film image of the same membrane resulted from NCS-1-specific immunolabeling. In both *A* and *B*, 1–12 are triplicate blots from different tissue samples. Six ''standard'' blots were made from the specially prepared human LDPFC homogenates with known amounts of the total protein. The background blots contain either pure homogenization buffer or BSA. (*C*) Typical film image produced by NCS-1 immunostaining of Western blot of human LDPFC. The image contains only a single band of ≈22 kDa, which is the molecular mass of NCS-1.

specially prepared ''standard'' homogenates of the human DLPFC containing a range of known amounts of total protein. Finally, each membrane included three slots made with pure tissue homogenization buffer and three slots made with BSA prepared as tissue samples, to control for nonspecific background staining.

Before immunostaining, each membrane was stained with SYPRO Rose Plus stain (Molecular Probes), which visualized the total protein within each slot blot. The staining was observed with a UV Photo Viewer Illumination System (Ultra-Lum, Claremont, CA) at a 365-nm wavelength (Fig. 1*A*). The fluorescent images of blots were digitized for densitometric analysis with a Universal Software (Advanced American Biotechnology, Fullerton, CA), and the total protein levels in sample slot blots were calculated by comparing the intensities of their staining with those of the above-mentioned six slots of the ''standard'' tissue homogenates. The immunostaining of the membranes included incubation with the primary chicken anti-frequenin antibodies (1:1,000 dilution; Rockland, Gilbertsville, PA) overnight at 4°C, which was followed by 1.5 h exposure at room temperature to horseradish peroxidase-conjugated donkey antichicken secondary antibodies (1:5,000 dilution; Jackson ImmunoResearch). Visualization of labeling was conducted with the Super Signal Chemiluminescence Substrate (Pierce) and X-Omat AR Film (Kodak). The specificity of this immunolabeling was evaluated by Western blots of three randomly selected samples from all cases used in the present study. As expected, the immunolabeling visualized a single 22-kDa band (Fig. 1*C*), which is the molecular mass of NCS-1 (13, 15). The generated film images of NCS-1-specific immunolabeling were digitized (Fig. 1*B*) and processed for densitometry, which assessed the levels NCS-1 in sample slot blots by comparing the gray value of the images of these blots with those of the six blots of the ''standard'' tissue homogenates. Levels of NCS-1 in the sample blots were calculated in equivalents of ng of the total protein in the blots of the ''standard'' homogenate producing the immunolabeling of the same intensity. Based on the estimations of total protein and the specific protein in each blot, we were able to express NCS-1 levels per nanogram of total protein in a given sample.

NCS-1 is a neuronal protein (11, 15, 16). This prompted us to try and express its levels per cortical neuron. Because it is not possible to determine precisely the number of neurons in the tissue samples used for blots, the closest representation of the quantity of a given protein per neuron is to express the levels of this protein per ng of neuronal DNA (35). For this purpose, we divided the mean levels of specific proteins in the protein slot blots from a given sample by the mean levels of DNA in the DNA slot blots from this sample and multiplied by the mean proportion of neurons in a section from the same tissue.

The DNA slot blots were produced as described (12, 35). They were generated on a set of Hybond-N Nylon membranes (Amersham Pharmacia) with every membrane containing slots of 12 samples in triplicates. As in the case of the blots for protein analysis, each of the 60 samples used in this study was blotted on three different membranes. All membranes also included a set of six ''standard'' blots containing a range of known amounts of salmon sperm DNA (Sigma), and six ''background'' slots of BSA. The slots were stained with SYBR DX DNA-specific Blot Stain (Molecular Probes). The staining was visualized at the wavelength of 254 nm and digitized. The digitized images were processed for densitometry and the amounts of DNA in sample blots were estimated by comparing the intensities of their staining with those of the above-mentioned six blots of the DNA standards.

The proportion of neurons in sections from each case was estimated by 3D cell counting as outlined in refs. 12 and 35. For each section, the counting was performed in five nonoverlapping randomly selected counting boxes (55-μm width × 8-μm depth) stretching across the entire thickness of the cerebral cortex from the pial surface to the white matter. The total number of cell nuclei and the number of neuronal nuclei were obtained for every counting box and then the mean proportion of neurons among the total cell nuclei was calculated for each case. Neuronal nuclei were identified based on the criteria of Selemon *et al.* (36). Cell counting was performed solely to determine the proportions of neurons in the tissue samples needed for calculation of the levels of specific proteins per neuronal DNA. The mean percentages of neuronal nuclei in sections of dorsolateral prefrontal cortex from schizophrenic, bipolar, and major depressive groups were within 5% of the mean proportion of neuronal nuclei in sections from the control

Table 1. Percentage of neurons in samples of the DLPFC from the Stanley Foundation Neuropathology Consortium

Groups	NC	SCHIZ	BPD	MD
% neurons among all cells ± SD	39.95 ± 7.4	44.62 ± 9.7	40.09 ± 8.9	42.01 ± 7.1
Differences between % of neurons in disease groups and control ± SD		4.67 ± 8.2	0.14 ± 7.4	2.06 ± 6.3

Each group consists of 15 cases.

Table 2. Effects of gender, hemisphere, most frequent causes of death, and alcohol consumption among the cases of the Stanley Foundation Neuropathology Consortium on NCS-1 levels in the DLPFC expressed per ng of neuronal DNA

Parameters	Groups	Mean ± SD	*P*
Gender	Males ($n = 36$)	24.24 ± 14.72	0.848 (*t* test)
	Females ($n = 24$)	23.56 ± 10.89	
Hemisphera	Right ($n = 27$)	24.13 ± 14.60	0.926 (*t* test)
	Left ($n = 33$)	23.80 ± 11.75	
Cause of death	Cardio/pulmonary ($n = 32$)	22.39 ± 13.69	0.808 (one-way ANOVA)
	Accident ($n = 4$)	22.95 ± 13.45	
	Suicide ($n = 20$)	24.87 ± 12.74	
Alcohol consumption at the time of death*	None/light ($n = 48$)	22.02 ± 11.77	0.619 (one-way ANOVA)
	Moderate ($n = 5$)	26.20 ± 9.94	
	Heavy ($n = 7$)	25.91 ± 16.03	

Note that the levels of calcyon are not affected by any of these parameters.
*Moderate alcohol use: one to two drinks a day; heavy alcohol use: more than one to two drinks a day; light alcohol use: less than one drink a day.

group (Table 1), and also in agreement with previous reports (37–39). Also in agreement with our previous study (12), haloperidol produced less than a 3% change in the proportion of neurons in the dorsolateral prefrontal cortex of monkeys treated with haloperidol relative to drug-naïve monkeys.

A statistical comparison of NCS-1 levels between different diagnostic groups was conducted by a one-way ANOVA followed by a Dunnett's post hoc comparison between controls and each of the mental disease groups. One-way ANOVAs were also used to evaluate whether levels of NCS-1 are influenced by cause of death (cardiopulmonary disease/accident/suicide) or alcohol consumption (no or light use/moderate use/heavy use; the definitions are given in Table 2). In addition, one-way ANOVA was used for comparison of the effects of treatments with antipsychotic, mood stabilizing, and a combination of antipsychotic/mood stabilizing drugs at the time of death. Correlation analysis was used for examination of possible influences of age, lifetime fluphenazine equivalent, postmortem interval, and brain pH. The effect of gender and hemisphere in the human cases and the effect of haloperidol treatment in nonhuman primates were assessed with two-tailed Student's *t* tests. Because, among all of the cases examined, all but four were Caucasians, no examination of the effects of race was performed. This study included too few non-alcohol drug abusers (with different individuals abusing different drugs) to allow a meaningful evaluation of the possible effects of drug abuse.

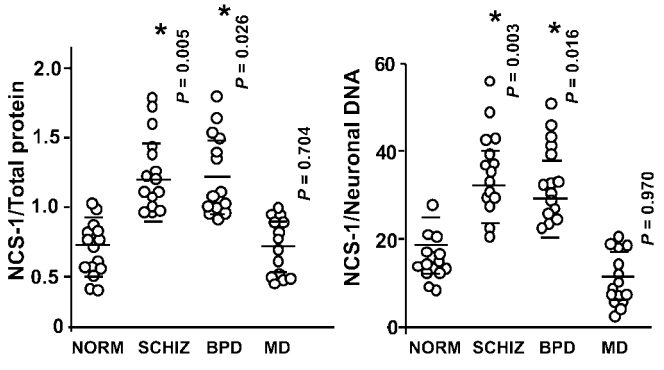

Fig. 2. Plots of the levels of NCS-1 (expressed per nanogram of total protein and per nanogram of neuronal DNA) in the DLPFC from NC, SCHIZ, BPD, and MD groups of the Stanley Foundation Neuropathology Consortium. Each group in the consortium = 15 cases. Circles represent the data from individual samples. The horizontal lines represent the mean values for the group. Vertical lines represent SD. *P* = statistical significance of Dunnett's post hoc one-way ANOVA comparison of the levels of NCS-1 between NC and a given mental disease group (*P* for the ANOVAs preceding the Dunnett's tests: 0.007 for NCS-1 expressed per total protein and 0.001 for NCS-1 expressed per neuronal DNA). The statistically significant increases in NCS-1 levels (marked by asterisks) as compared with controls are seen in SCHIZ and BPD groups.

Results and Discussion

Increase of NCS-1 in DLPFC of SCHIZ and BPD Patients. We found that the DLPFC of SCHIZ and BPD patients displayed >50% higher levels of NCS-1 compared with the same cortical region of NC individuals (Fig. 2). This increase was observed regardless of whether the data were expressed per total tissue protein or per neuronal DNA, which suggests that they are likely to reflect a specific up-regulation of this protein, rather than nonspecific difference in total protein or neuronal density between the groups examined in this study. In contrast, no significant differences were detected in DLPFC levels of NCS-1 between NC and MD groups (Fig. 2).

To determine whether the observed elevations of NCS-1 levels in SCHIZ and BPD groups were related to the effect of medications, we compared the levels of NCS-1 in SCHIZ and BPD patients maintained at the time of death on antipsychotic and/or mood stabilizing drugs with the levels of this protein in drug-free cases from the same two groups. This comparison was

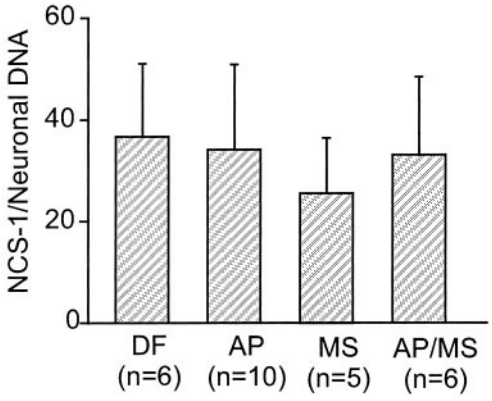

Fig. 3. Histogram showing the DLPFC levels of NCS-1 in SCHIZ and BPD patients from the Stanley Foundation Neuropathology Consortium who at the time of death received either no medications, antipsychotic drugs, mood-stabilizing drugs, or a combination of these drugs. Each column represents the mean ± SD. No statistically significant differences are present (*P* of one-way ANOVA = 0.407). DF, drug free; AS, antipsychotic medication; MS, mood-stabilizing medications.

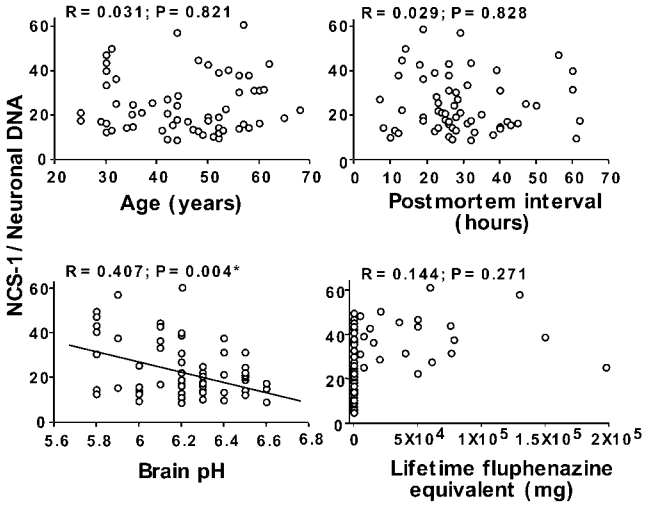

Fig. 4. Plots of correlation analyses of the levels of NCS-1 in the DLPFC expressed per neuronal DNA in relation to age, postmortem period (PMI), brain pH, and the lifetime dosage of antipsychotic drugs in fluphenazine equivalents among the cases constituting the Stanley Foundation Neuropathology Consortium. Circles represent values for individual samples. R = correlation coefficient; P = significance. There are no significant correlations between NCS-1 levels and age, PMI, brain pH, and the lifetime dosage of antipsychotic drugs. In contrast, there is a significant negative correlation between the levels of NCS-1 and tissue alkalinity (indicated by the descending line and the asterisk near the appropriate *P* value).

not statistically significant, indicating that the observed NCS-1 abnormalities were not caused by medication (Fig. 3). Furthermore, comparison of NCS-1 levels in DLPFC of drug-naïve and chronic haloperidol-treated monkeys failed to reveal significant differences (level of NCS-1 per neuronal DNA ± SD in haloperidol-treated monkeys was 21.26 ± 4.03, whereas in control animals this level was 19.43 ± 3.53; *P* of two-tailed *t* test is 0.747). In addition, we found that NCS-1 levels were not affected by gender, hemisphere, causes of death, or by alcohol consumption (Table 2). Furthermore, levels of this protein were not correlated with lifetime consumption of antipsychotic medications (in fluphenazine equivalents), age of the individuals included in the study, and the length of the postmortem interval (Fig. 4). Other than diagnosis, the only additional parameter predictive of changes in NCS-1 was the pH of the brain tissue, with the levels of this protein being negatively correlated with increase in tissue alkalinity (Fig. 4). This, however, does not negate the role of diagnosis as a predictor of NCS-1 levels because, as was mentioned earlier, the diagnostic groups used in this study were matched for values of brain pH.

Possible Consequence of Increase in NCS-1. The observation that SCHIZ and BPD patients display similar changes in NCS-1 is in agreement with the recent observation of Knable *et al.* (33) that these two disorders seem to share many biochemical abnormalities. Presumably, this is a reflection of certain overlap of symptomatic clusters constituting SCHIZ and BPD diagnoses (40, 41). It is also notable that, in contrast to the majority of reports of decreased expression of specific markers in brains of SCHIZ and BPD patients (33, 42), our findings point to a significant increase in the levels of NCS-1 in these two diseases. It is tempting to speculate that such an increase indicates that NCS-1 is actively involved in producing the SCHIZ- and BPD-associated disorganization of brain functionality.

As mentioned, NCS-1 attracted our attention as a DRIP involved in suppression of desensitization of DR2s (11), which decreases the rate of deactivation and internalization of these receptors and, thus, enhances their influence on cell activity (43). Based on DR2 involvement in inhibition of DLPFC activity (44–46), the observed NCS-1 up-regulation might be expected to result in decreased activation of the DLPFC in SCHIZ and BPD patients. Furthermore, this could occur without any detectable change in the level of DR2s. This prediction closely matches the observations of decreased activity of the DLPFC in SCHIZ and BPD (47, 48) without detectable alterations in the levels of DR2 sites in this region (49).

Non-DRIP actions of NCS-1 also need to be considered. It is possible that the over-expression of this protein may affect signal processing in the DLPFC in SCHIZ and BPD patients by promoting the A-type K^+ current (25), affecting the activity of P/Q-type Ca^{2+} channels (26, 27) and facilitating synaptic neurotransmitter release (20, 22). An increase in the K^+ current involved in membrane outward rectification would prevent DLPFC neurons from reaching firing threshold (50). Similar to the over-activation of DR2, this would result in depression of DLPFC activity. There have been reports of both NCS-1-induced decreases (26) and NCS-1-induced increases (27) in the activity of P/Q-type Ca^{2+} channels. Nevertheless, any change in Ca^{2+} current would interfere with the normal functional activity of the DLPFC. As far as neurotransmission is concerned, the overall functional consequence of NCS-1 up-regulation would depend on the balance of the resultant elevations in the release of inhibitory and excitatory neurotransmitters in the DLPFC of SCHIZ and BPD patients. It is also interesting that a possible NCS-1 up-regulation-associated increase in neurotransmission in the DLPFC of SCHIZ patients would take place on the background of a deficit in neuropil, which likely is associated with decline in neuronal connectivity (51–54). In this case, NCS-1 up-regulation may in part provide a compensatory response for the SCHIZ-induced neuronal connectivity decline. Up-regulation of NCS-1 may also interfere with posttranslational trafficking of certain proteins, which would affect the intracellular positioning and, ultimately, the levels of these proteins (23, 24). Abnormal positioning and/or levels have been reported for numerous synaptic, cytoskeletal, and other proteins in the DLPFC of SCHIZ and BPD patients. It is possible that some of these changes are related to NCS-1 up-regulation.

SCHIZ and BPD as Consequences of Disturbances in Ca^{2+} Signaling. The present study not only supports our original hypothesis that SCHIZ and BPD may be associated with alterations in DRIPs, but also adds NCS-1 to a growing list of Ca^{2+}-binding proteins, such as visinin-like protein-1, Ca^{2+}-calmodulin-dependent kinase II, calbindin, parvalbumin, and S100B, which have been shown to exhibit abnormal expression in the brain of SCHIZ and/or BPD patients (55–61). In addition, in SCHIZ brains, abnormalities have been detected in Ca^{2+}-stimulated phosphoinositide turnover, Ca^{2+} channels (33), and the Ca^{2+}-release-activating DRIP, calcyon (12), whereas altered levels of Ca^{2+} were described in lymphoblasts of BPD patients (62, 63). Such findings point to an intriguing possibility: although SCHIZ and BPD can be induced by multifactorial causes, these causes may eventually disturb Ca^{2+} signaling, which, in this case, would constitute the central mechanism underlying abnormal brain functionality in both diseases. The differences between SCHIZ and BPD might then be determined by constellations of abnormalities in Ca^{2+}-associated intracellular cascades.

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