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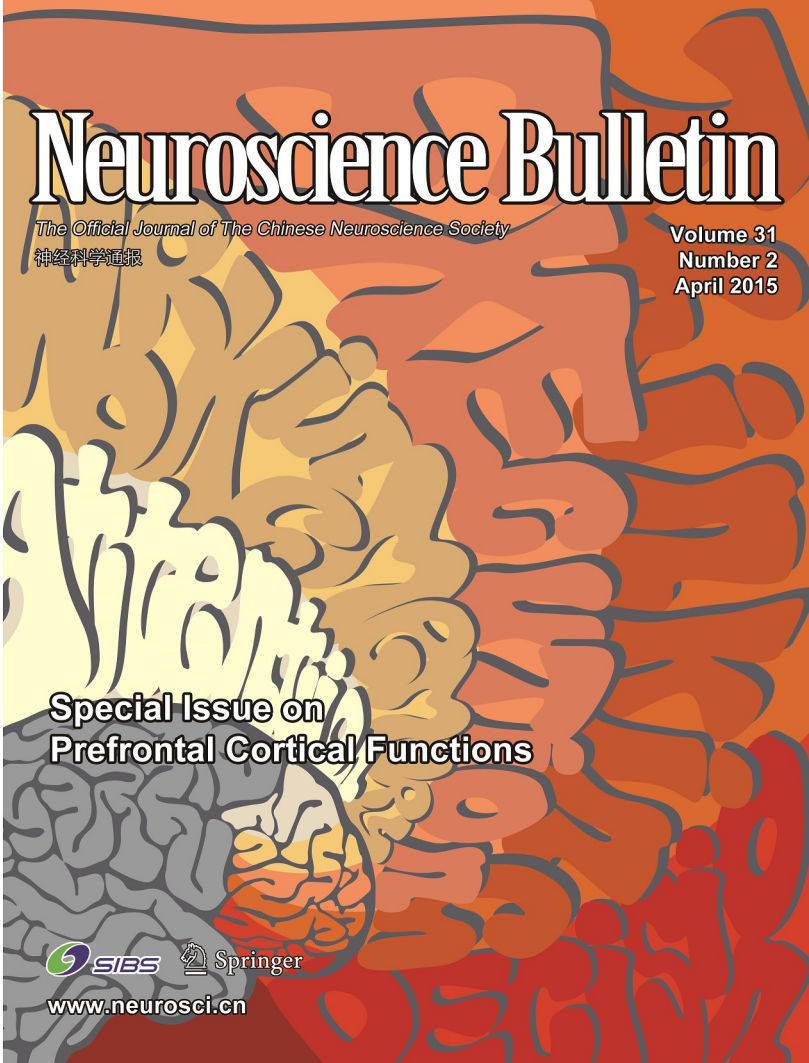

Special Issue on Prefrontal Cortical Functions

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About the Cover

It is generally believed that there is an integral link between a person's personality and the functions of the prefrontal cortex (PFC), such as the regulation of attention, working memory, thinking, execution, and decision-making. In this special issue, we present a collection of articles that discuss current knowledge of how these functions are regulated in the PFC. On the cover image, artistically illustrated terms for the main PFC functions swirl through the cortical folds. Cover art by Dr. Yefei Li.

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·**Editorial**·

A step forward in the understanding of prefrontal cortical functions

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The prefrontal cortex (PFC) is an integrative area that guides behaviors and thoughts[1-3]. During the past century, a great many studies have been performed on PFC functions, using a variety of technical approaches such as anatomy, lesion, neuropsychology, electrophysiology, and functional imaging[2, 3]. It is well known that the PFC plays an essential role in inhibitory control of behavior, regulation of attention, working memory, decision-making, and behavioral organization[1-6].

In October 2013, the *4th International Conference on Prefrontal Cortex* was held in Nanchang University (Nanchang, China), co-organized by Bao-Ming Li (Nanchang University, China), Shintaro Funahashi (Kyoto University, Japan), Yong-Di Zhou (East China Normal University, China), and Satoru Otani (Ryotokuji University, Japan). A group of scientists from China, Japan, the USA, Germany, and Brazil comprehensively introduced their studies and extensively discussed progress in the understanding of PFC functions. This special issue covers the reviews and reports by the speakers at the conference.

Evolutionarily, the PFC is the last-emerged structure of the brain. It is unknown how it has evolved into such a complex system in humans. The mesocortical dopamine projection into the PFC is essential for its cognitive functions. Lee and Goto propose a hypothesis that the mesocortical dopamine projection is one of the biological substrates involved in PFC evolution, and that this evolutionary process may result in the emergence of psychiatric disorders such as schizophrenia[7].

Working memory refers to the cognitive process of maintaining information for seconds for subsequent goal-directed actions. Persistent neuronal firing in the sensory cortex and the PFC during the delay period in a working memory task has been considered to be the neuronal substrate for working memory maintenance. However, the role of the delay activity in the sensory cortex is thought to differ from that in the PFC. Ku *et al*. propose that the delay activities in the sensory cortex and the PFC reflect the quality and quantity of representations in working memory, respectively[8].

In addition to maintaining working memory information, the PFC is thought to store memory traces for “rules” or “strategies” that determine the temporal structure of behavior, and this kind of memory might be served by synaptic plasticity in the PFC. Otani *et al*. review the induction of long-term synaptic plasticity in the medial PFC of rats, with special emphasis on the regulation of synaptic plasticity by dopamine. They conclude that synaptic plasticity in the PFC is powerfully modulated by dopamine in an inverted-U-shaped, dose-dependent manner[9].

Cognitive disorders such as schizophrenia are associated with dysfunction of the PFC, and with changes in N-methyl-*D*-aspartate receptors (NMDARs). The dorsolateral PFC, which is highly evolved in primates, subserves the higher cognitive functions especially affected in mental disorders such as schizophrenia. Wang and Arnsten review new evidence that demonstrates a key role of NR2B-containing NMDARs in the cognitive functions of the dorsolateral PFC, especially spatial working memory[10].

Imaging techniques such as functional magnetic resonance imaging, positron emission tomography, and encephalography have been widely used to reveal

the changes in brain activity associated with functions. However, it is difficult to draw conclusion about the causal relationships between them. Non-invasive electrical stimulation with direct or alternating currents allows the manipulation of brain activity and excitability and helps to uncover the causal relationships between brain activities and functions. Kuo and Nitsche review the principal mechanism of this approach and its application in exploring PFC functions[11].

It is hypothesized that schizophrenia results from disrupted brain connectivity. It is important to know whether there is anatomical and functional dysconnectivity between the PFC and other brain regions, and how such dysconnectivity is linked to schizophrenia. Imaging techniques, such as diffusion tensor imaging and functional magnetic resonance imaging, make it possible to explore these issues. Zhou *et al.* review the recent progress in understanding the anatomical and functional dysconnectivity of the PFC in humans and their implications in schizophrenia[12].

Much is understood about the interactions between cognitive functions, from language to cognitive control, from attention to memory. However, it has been difficult to dissociate these functions because their representative cortical regions are located in close proximity. Cai and Van der Haegen review a series of studies investigating the relationship between language and other cognitive functions by examining their functional lateralization. The authors argue that the hemispheric lateralization of language and its link with handedness could offer an appropriate starting point to shed light on the relationships between different cognitive functions[13].

It is known that there is a close relationship between PFC dysfunctions and the symptoms of schizophrenia. In addition to typical features such as hallucinations, delusions, and withdrawal from social activities, schizophrenic patients present with many forms of cognitive disorders. For many years, there has been no suitable non-human primate model of schizophrenia. Mao *et al.* report a monkey model of this disease, which is induced by treatment with phencyclidine, a non-specific competitive NMDAR antagonist, and the symptoms of schizophrenia can be ameliorated by atypical antipsychotic drugs such as clozapine[14].

Attention deficit and hyperactivity disorder (ADHD) is a syndrome characterized by inattention, impulsivity, and/or hyperactivity, and seriously affects the cognitive development of children. The lack of suitable animal models limits the development of new medications for ADHD. Ma *et al.* review the previous studies in nonhuman primates showing that blockade of PFC α2A adrenoceptors mimics the major symptoms of ADHD, providing new insights for developing novel animal models, and contributing to the understanding of the neurobiological basis of ADHD[15].

This special issue also includes a Research Highlight[16], introducing the work published in *Science* by Chengyu Li's laboratory at the Institute of Neuroscience, Chinese Academy of Sciences[17]. As noted above, working memory involves a short-term interval of retention termed the 'delay period'. It is known that neurons in the medial PFC of rodents (thought to be homologous to the dorsolateral PFC in monkeys) demonstrate elevated delay-period activity, but its functional significance is unclear. Li's group optogenetically suppressed or enhanced the delay activity of pyramidal neurons in the medial PFC of mice. They found that behavioral performance was impaired during the learning stage of the working memory task (olfactory delayed-nonmatch-to-sample go/no-go task), but not after the mice were well-trained, indicating that the delay-period medial PFC activity is involved in learning the task, but not in the maintenance of working memory information. They conclude that properly-regulated delay-period medial PFC activity is critical for information retention when mice are learning the working memory task, but not for working memory maintenance after the animals are well-trained[17].

We anticipate that this special issue will provoke further studies, providing a better understanding of the cognitive functions of the prefrontal cortex, and eventually lead to more effective treatments of prefrontal cortical cognitive disorders.

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·Review·

Prefrontal cortical dopamine from an evolutionary perspective

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In this article, we propose the hypothesis that the prefrontal cortex (PFC) acquired neotenic development as a consequence of mesocortical dopamine (DA) innervation, which in turn drove evolution of the PFC into becoming a complex functional system. Accordingly, from the evolutionary perspective, decreased DA signaling in the PFC associated with such adverse conditions as chronic stress may be considered as an environmental adaptation strategy. Psychiatric disorders such as schizophrenia and attention deficit/hyperactivity disorder may also be understood as environmental adaptation or a by-product of such a process that has emerged through evolution in humans. To investigate the evolutionary perspective of DA signaling in the PFC, domestic animals such as dogs may be a useful model.

Keywords: neurodevelopment; neoteny; psychiatric disorder; stress; animal model; synaptic plasticity; environmental adaptation

Introduction

The prefrontal cortex (PFC) is one of the central brain regions that mediate higher cognitive and affective functions[1, 2]. Extensive investigations have unveiled the mechanisms of neuronal network information-processing as well as the genetic and molecular machinery underlying PFC functions. The PFC is also suggested to be evolutionarily the latest component of brain structure, and therefore it plays the most important role in organizing human-specific behavioral traits[1, 3, 4]. Nonetheless, how the PFC evolved into the complex system in humans remains unclear.

The mesocortical dopamine (DA) projection into the PFC from the ventral tegmental area of the midbrain is essential for PFC functions (for an extensive review, see Seamans and Yang[5]). In this article, we propose the hypothesis that the mesocortical DA projection into the PFC is one of the biological substrates involved in the evolution of the PFC. Moreover, this evolutionary process may have resulted in the emergence of psychiatric disorders such as schizophrenia and attention deficit/hyperactivity disorder (ADHD).

Mesocortical DA Regulation of PFC Development

DA innervation of the cortex is more restricted than those of serotonin (5HT) and norepinephrine (NE). NE projections arising from the locus coeruleus and 5HT projections from the dorsal raphe cover almost the entire cortex, from the frontal cortical area to the posterior parts including the visual cortex[6]. In contrast, DA projections are distributed primarily to the frontal and temporal cortex, and other cortical areas lack this innervation[7].

The development and maturation of the cortical areas innervated by DA appear to be slower than other cortical areas lacking DA innervation. A human imaging study has shown that development of the frontal and temporal cortex continues into young adulthood, much later than in other cortical areas[8]. Accordingly, DA receptor expression (both D1 and D2) changes dynamically until early adulthood[9], and this coincides with maturation of the PFC. Such

PFC DA receptor expression dynamics may impact PFC development (e.g. delaying developmental processes if DA signaling affects developmental timing or rate). In addition, the N-methyl-*D*-aspartate (NMDA) receptor has also been suggested to play an important role in neuronal development[10]. Recent studies have shown that the DA receptor is functionally and physically[11, 12] coupled to the NMDA receptor. Thus, lower DA receptor expression during early development could also influence NMDA receptor-dependent developmental processes. Such observations provide the insight that there may be a relationship between delayed cortical development/maturation and the DA projection to the cortex.

Several lines of evidence suggest that DA signaling influences PFC development. First, Lumbe and colleagues examined the development of DA and serotonin (5HT) projections into the PFC in non-human primates at different ages using immunohistological staining to assess the density of fibers containing tyrosine hydroxylase and 5HT that contact PFC pyramidal neurons[13]. They found that the DA projection does not fully mature until early adulthood. In contrast, the 5HT projection is already mature at an early developmental stage. These findings suggest that DA development is an important factor that may determine the timing of PFC development and maturation.

Studies have also investigated the effects of neonatal DA depletion by 6-hydroxydopamine injection into the PFC of rodents. Kalsbeek and colleagues reported that PFC pyramidal neurons in adult rats with neonatal DA depletion are smaller with shorter dendritic lengths[14]. Such morphological changes can be interpreted in two ways: one is that the morphology of mature PFC pyramidal neurons in adult animals that develop without DA signaling resembles that of immature neurons; and the other is that such morphological changes may indicate atrophy of the fully mature form of neurons. However, the latter interpretation is unlikely, since DA depletion is given in the neonatal period before the proliferative growth of neurons. In addition, the PFC volume in rats with neonatal DA depletion is larger than that of normal rats[15], contradictory to the case of atrophy in which shrinkage of neurons and thereby smaller volume are expected, although such enlargement of the volume may be attributed to the proliferation of glial cells, not neurons.

Decreased mesocortical DA transmission has been suggested in the pathophysiology of ADHD[16]. Then, if DA signaling affects PFC development, alterations of its developmental rate would be expected in ADHD children. A magnetic resonance imaging study of ADHD children[17] has revealed that development of the gray matter volume in the PFC is significantly delayed compared to controls. This finding further supports the hypothesis that DA is a biological substrate that promotes PFC development.

Several studies have shown that DA and stimulation of DA receptors facilitate neuronal growth[18-20]. However, these studies used striatal neurons; so, such molecular mechanisms of DA-dependent facilitation of neuronal growth may not necessarily be applicable to PFC neurons. Nevertheless, these studies still support the idea that DA signaling is involved in the regulation of neuronal development.

Collectively, mesocortical DA may play an important role in the PFC developmental process, particularly in regulation of the timing and rate of development and maturation. Thus, PFC development and maturation may be delayed as a consequence of DA innervation, which does not mature until adulthood (Fig. 1). On the other hand, the cortical areas lacking DA innervation develop and mature faster than the PFC. However, this does not mean that DA is the sole determinant of developmental processes in the PFC. Indeed, it is possible that other neurotransmitters are also involved.

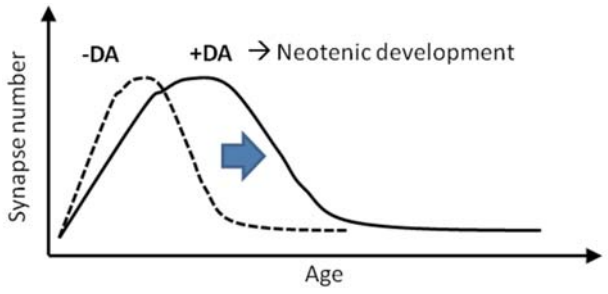

Fig. 1. Schematic illustrating delayed PFC development and maturation in terms of synaptogenesis and elimination of pyramidal neurons with mesocortical DA signaling. In PFC pyramidal neurons, the synapse number increases with growth. Then, during adolescence, synaptic elimination takes place to refine the neuronal networks[22]. This process is delayed with DA innervation, which continues in the PFC until adulthood.

Neotenic PFC Development with DA Signaling

Based on the above evidence, we propose the hypothesis that mesocortical DA signaling is an important biological substrate for evolution of the PFC, enabling neotenic development.

Neoteny is the retention of juvenile traits in adults, and is thought to be an important factor in evolution, as it provides greater flexibility in adapting to the environment by delaying the development of organs for specific functions[21]. Indeed, the sexual maturation of humans takes up to two decades, much longer than in any other animal[22].

The development and maturation of the PFC in terms of synaptic spine density on dendrites of pyramidal neurons have been shown to continue until early adulthood in humans[23], non-human primates[24], and rodents[25]. A more recent study has suggested that the maturational process of the PFC could be even longer, continuing until the thirties in humans[26]. Therefore, neotenic development is one of the most characteristic features of the PFC. Such delayed development may have enabled the PFC to evolve into a highly complex system to mediate an assortment of cognitive and affective functions.

In order to establish this hypothesis, an intrinsic correlation between evolutionary and developmental processes is required. Indeed, it has been suggested that evolution and development have strong relationships, as reflected in the field of evolutionary developmental biology or "evo-devo"[27]. One of the key concepts of evo-devo is heterochrony, which refers to changes in the timing and rate of development of organisms, including neoteny. Genetic (e.g. homeobox genes) and morphological evidence that supports the critical roles of such heterochronic processes in evolution has been reported[28-33].

Reduced PFC DA Signaling as an Environmental Adaptation Strategy in Evolution

Our hypothesis predicts that mesocortical DA signaling plays an important role in neotenic development, and thereby the evolution of the PFC, especially when DA signaling is lower than normal. In this regard, it is important to note that although decreased DA signaling is usually considered deficient and abnormal from the perspective of conventional neuroscience, it could be advantageous from the evolutionary perspective. How could decreased DA signaling in the PFC, which has been shown to cause cognitive and affective dysfunction [5, 34], be advantageous?

Stress alters DA release in the PFC in rodents and primates[35]. Although brief, acute stress temporarily increases DA release in the PFC[36], more severe, chronic stress decreases basal DA tone regardless of the stress type (e.g. restraint[37] or cold[38]) in adult animals. However, the effects of chronic stress on PFC DA release in the adult brain may differ from that in the developing brain. For instance, Giovanoli and colleagues[39] recently reported that exposure of adolescents to chronic stress affects 5HT, but not DA, in the PFC. This study provides additional insights into our hypothesis. First, this study assessed the content but not the release of DA and 5HT in the PFC. The absence of a change in DA concentration in the PFC with stress suggests that chronic stress affects the release but not the synthesis of DA. In addition, in this study, the effects of chronic stress in adolescence were examined in adulthood; but the effects of stress may not be persistent, but could be reversible and recover with time. Therefore, delayed PFC development with decreased DA release may be evident only when organisms are under chronic stress, and once the situation improves, the developmental rate may return to normal. Finally and most importantly, this study used variable stress procedures (5 different stressors, applied on alternate days, between postnatal day 30 and 40). Stress-induced changes often differ depending on the environment in which stress is generated. For instance, chronic restraint but not unpredictable stress induces amygdala-dependent brain and behavioral changes[40]. This suggests that the environment in which stress is generated determines how the stress-induced changes take place, whereas stress (e.g., stress hormones) and associated changes such as decreased DA release may rather signal or trigger subsequent environment-dependent changes. The unpredictable stress procedure was probably developed for animal studies, given that animals tend to show habituation against repeated exposure to identical stressors, making unpredictable stress more convenient in the experimental setting. However, it is important to note that it is uncommon in real life (both in humans and animals) for stressful environments to change rapidly from day to day. In our hypothesis, an adaptation process would take place under

a particular environment that causes stress for a prolonged period. Therefore, even if the stress exposure is sufficiently long, adaptation may not occur when the environment is rapidly changing.

Many studies have been conducted to reveal *how* chronic stress decreases DA signaling in the PFC (i.e. Tinbergen's proximate view, which questions a mechanism of function of a system[41]), and many of the molecular and cellular mechanisms underlying this process are known[34]. In contrast, *why* DA signaling is decreased in the PFC by chronic stress (i.e. Tinbergen's ultimate view, which questions an evolutionary origin of function of a system[41]) has not yet been explored. However, there must be a reason that DA signaling in the PFC is decreased by chronic stress.

Rodents and primates are estimated to have diverged ~100 million years ago[42]. Given that chronic stress induces similar, if not identical, cognitive and affective deficits as well as associated brain changes such as decreased DA signaling both in rodents and primates, these "disadvantageous" phenotypes were already present in mammals before this divergence, and have been maintained for >100 million years. Darwinian evolutionary theory (natural selection)[43] suggests that, if the chronic stress-induced decrease in DA signaling in the PFC and the consequent changes in PFC-dependent cognitive and affective processes were disadvantageous for survival, such phenotypes should have vanished. Therefore, even though decreased DA signaling and altered PFC function have major disadvantages in the normal environment, such phenotypes may still be advantageous for survival in specific environments that cause chronic stress.

If decreased DA release delays PFC development, then chronic stress during development would also delay PFC maturation. There is no direct evidence to support this idea, and further investigation is needed. However, there are reports that children exposed to stress due to family problems exhibit delayed physical[44] and mental[45] development, indirectly supporting our hypothesis.

If chronic stress and associated brain changes have played a role in the evolution of the brain, such stress-induced alterations should be inheritable. A brief report of transgenerational inheritance of stress-induced alterations appeared in 1970[46]. However, this study was largely ignored until recently when the transgenerational inheritance of epigenetic changes was confirmed. Behavioral changes caused by neonatal[47] or adult[48] exposure to stress have been recently reported again, confirming that stress-induced changes are inheritable. It appears that the inheritance of these environmentally-induced (or acquired) phenotypes involves epigenetic mechanisms[47, 48]. Since these studies have followed two or three generations at most, it is still unclear whether such stress-induced epigenetic changes can be translated into equivalent genetic changes for inheritance across generations. Nevertheless, domestication processes in chicken[49] and silkworm[50] have been reported to involve epigenetic inheritance. Importantly, these studies raise the question of why stress-induced changes, which are thought to be "deficits" and therefore disadvantageous phenotypes, are inherited and the biological mechanisms mediating them are still found in organisms.

Taken together, chronic stress could be a driving force for PFC evolution, and the consequent decrease of DA signaling may provide greater flexibility to the PFC for developing into a more complex system with neotenic development to adapt to or overcome severe environmental conditions (Fig. 2).

Psychiatric Disorders Associated with Lower PFC DA Signaling

Decreased PFC DA release has been implicated in psychiatric disorders such as ADHD[51] and schizophrenia[52]. The third implication of our hypothesis is that such disorders may not necessarily be considered deficits, but could rather be understood as an environmental adaptation strategy (i.e. ADHD) or a by-product of adaptation (i.e. schizophrenia) that has emerged through evolution in humans.

ADHD

ADHD is one example of a psychiatric condition that could illustrate the beneficial effects of decreased DA signaling in the PFC. ADHD is a childhood-onset condition with core symptoms consisting of hyperactivity, impulsivity, and attention deficit (short attention span). These behaviors have been associated with decreased DA release in the PFC in animal studies[53-55]. Although these symptoms

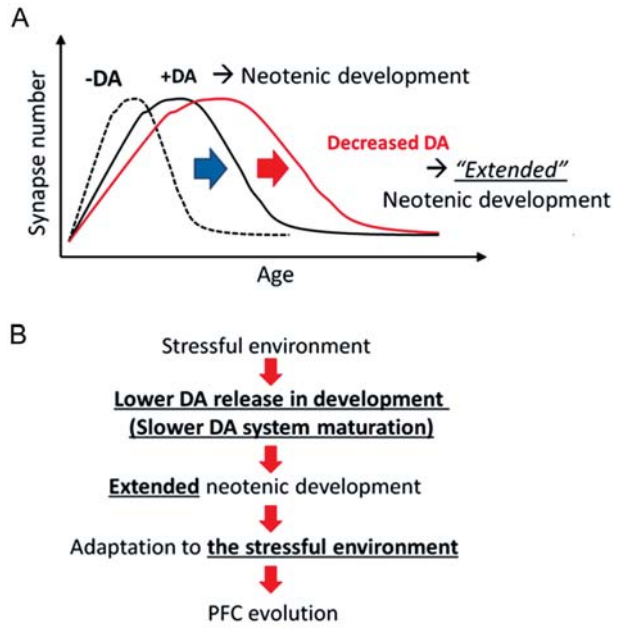

Fig. 2. Diagrams illustrating the process that, from an evolutionary perspective, lower mesocortical DA may play a beneficial role in the PFC. A: Schematic of the extension of delayed PFC development and maturation expected with lower DA signaling during the developmental process. B: Flowchart of the hypothetical evolutionary process of the PFC with mesocortical DA signaling driven by severe, chronic stress.

are problematic in modern human society such as school life, and therefore have to be treated, they still yield clear advantages for survival in severely stressful environmental conditions where life is threatened[56]. Hyperactivity enables constant exploration of the environment for the faster detection of threats. Similarly, attention deficit with inability to sustain attention to a particular target enables shifting of attention and scanning from one object to another to monitor threats. Impulsivity also allows rapid responses to escape from dangers.

How delayed maturation with decreased DA signaling in the PFC is associated with ADHD is still unclear. For instance, in animals living in the wild, faster maturation appears to be advantageous to escape from predators. Nevertheless, ADHD-like behavioral changes with decreased DA release in the PFC can be considered as a trade-off mechanism, such that delayed maturation exposes animals to more dangers, but ADHD-like behavioral changes reduces them.

Schizophrenia

Reduced DA function in the PFC has been suggested in schizophrenia[52]. In addition, stress appears to be an important factor, such that stress exposure often precedes the first episode of symptoms as well as exacerbating or precipitating symptoms[57, 58].

Various environmental and social conditions can be sources of stress. From the conventional viewpoint, environmental and social enrichment is usually considered beneficial, whereas a poor environment and social isolation are deficient for social organisms. However, it is important to note that exaggerated environmental and social enrichment can also be stressful. For instance, people living in cities, where the environmental complexity is great, are exposed to stronger stress than those living in rural areas[59]. Commuters during rush hours also suffer high-intensity stress[60]. Therefore, crowding within a group can be stressful to social organisms. Stress with excessive social crowding is particularly interesting, since it can generate evolutionary pressure to cope with such conditions, by developing an adaptation strategy to better assess the behavior or mental states of others, i.e. a theory of mind (ToM)[61, 62]. Indeed, PFC activity has been shown to be associated with ToM[63]. Mirror neurons, which become active during both motor action and the observation of such action by others, are found in the PFC[64]. The mirror neuron circuit, involving the PFC and DA release in this region, plays an important role in understanding the behavior and emotional states of others[65]. Therefore, one scenario is that stress with overcrowding in a tribe may cause decreased DA release in the PFC, which is a signal or trigger to develop a strategy, ToM, as an adaptation to this specific stressful environment through evolution.

In this process, decreased DA may be just a signal or trigger for adaptation by delaying development, and thereby providing a longer adaptation period. Therefore, delayed development by itself does not yield specific adaptation. How adaptation takes places depends on the specific environment, such that adaptation could involve as many changes as there are stressful environments to which organisms are required to adapt. Indeed, stress is associated with various environmental factors (sych as social isolation, social crowding, social defeat, physical pain, and restraint), such that adaptation to stressful environments

does not involve only one change, but can include as many changes as there are different stressful environments. How the system changes for adaptation depends on the types of stressors. Therefore, adaptation to stressful environments can vary, and the psychiatric conditions that emerge from such processes could also be variable and different. The evolution of such an adaptation strategy most likely needs multiple generations. Thus, this argument does not imply that organisms with decreased PFC DA release immediately develop advantageous strategies, including ToM, as adaptive responses to stressful environments.

ToM deficits have been reported in schizophrenia[66, 67] and autism[61]. Nevertheless, the bases of these deficits may differ between the disorders. Crespi and Badcock have suggested that schizophrenia and autism are diametrically opposite psychiatric conditions, autism associated with underdeveloped ToM, and schizophrenia associated with overdeveloped ToM[68]. Overdeveloped ToM can passably explain the positive symptoms of schizophrenia such as paranoia and delusions. Schizophrenic patients often claim that they are controlled by TV, or neighbors are spying on them. These claims illustrate that patients peculiarly find that inorganic objects have minds like living organisms, or have a distorted understanding of the mental state of others.

Some of the schizophrenic symptoms may emerge as a by-product of the evolutionary process of ToM function as an adaptation strategy for stress associated with overcrowding within a group. This speculation is also consistent with the evolutionary psychiatric hypotheses proposed by Burns that schizophrenia emerged as a by-product in the evolution of human social behavior[69], by Stevens and Price that schizophrenia is an advantageous evolutionary phenotype playing the role of splitting a group when the population becomes too large[70], and by Saugstad that the less clear cerebral lateralization in schizophrenia is associated with late, slow maturation of the cortex[71]. On the other hand, social isolation can also be stressful, which could generate an evolutionary pressure for an adaptation strategy against isolation. Such evolutionary pressure may be the basis of the autistic phenotype.

Predictions and Experimental Approaches

Predictions Based on the Hypothesis

Our hypothesis suggests that cortical areas such as the visual cortex where DA innervation does not develop mature faster than cortical areas such as the PFC with DA innervation, since mesocortical DA signaling is involved in the development of the PFC neuronal network, but the mesocortical DA system does not mature until adulthood. Development of the PFC consequently continues until adulthood when the mesocortical DA system also matures. Our hypothesis therefore predicts that PFC development and maturation are accelerated by the augmentation of mesocortical DA signaling (e.g. pharmacological treatment such as psychostimulants). In contrast, PFC development and maturation are extraordinarily delayed by the attenuation of mesocortical DA signaling (e.g. pathological changes suggested in ADHD delayed development of the PFC[17] or DA fiber depletion by 6-OHDA).

Such a prediction appears to be partly supported by recent studies suggesting that addictive drugs that increase DA release accelerate aging[72-77]. These studies have shown that chronic abusers of amphetamines, cocaine, and alcohol exhibit cognitive decline and greater cortical atrophy indicative of accelerated aging. A study by Cheng and colleagues has shown that heroin abusers exhibit lower telomerase activity, which results in shorter telomeres, the biological marker of aging. Moreover, such lower telomerase activity is correlated with PFC gray and white matter thickness in abusers[78]. The possibility of accelerated aging by DA agonists is also interesting in relation to ADHD treatments in which DA agonists such as methylphenidate have been used. Given that ADHD may involve delayed development[17], the therapeutic effects of DA agonists in ADHD may be achieved not only by increased DA transmission, but also by accelerating aging. Indeed, development and aging could be distinct processes, and therefore the mechanisms involved in aging may not necessarily be similar to the mechanisms involved in neuronal development and maturation.

Empirical Approach

Several experimental approaches may confirm or refute the hypothesis. Many studies have already investigated the effects of physical, pharmacological, and psychological manipulations that alter PFC DA release on PFC function. However, very few have examined the impact of these manipulations on PFC development. Thus, investigations focusing on changes in the developmental trajectory of

PFC neuronal networks (timing and speed of synaptic growth and pruning, which could be measured by dendritic spine quantification[79] or expression of synapse-associated molecules such as synaptophysin[80]) caused by manipulations such as local DA depletion with microinfusion of 6-OHDA into the PFC, chronic exposure to stress (e.g. maternal deprivation[81]), and repeated psychostimulant administration given at an early stage of development in animals, would be promising for testing the hypothesis.

Domestic Animals: a Model of PFC Evolution with DA

An alternative approach to investigating the evolutionary role of mesocortical DA in the PFC may lie in the use of domestic animals.

Domestication is a process of selective breeding that reduces aggression and facilitates sociality in animals due to decreased intra- and inter-species competition for resources. Such domestication is not necessarily achieved by human hands only, but could be a self-generated, i.e. self-domestication, as has been suggested in bonobos[82]. In particular, domesticated animals exhibit neotenic features (delayed development, maintaining juvenile morphological features into adulthood) and higher cognitive function and sociality, which are associated with PFC activity, than wild animals[82]. In particular, domesticated animals include laboratory rodents (e.g. ICR mice, Sprague-Dawley rats), which are commonly used in biomedical research. It is possible that PFC function and associated DA transmission in the PFC differ significantly between laboratory mice/rats and wild rodents (e.g. MSM mice). Comparison between laboratory and wild rodents for associations between cognitive function and social behavior and PFC neuronal network morphology, plasticity, volume, and DA levels would be promising. In support of this approach, a recent study by Takahashi and colleagues has reported differences in social behavior between laboratory and wild mice[83].

The use of dogs as a model for understanding human social behavior has been proposed[84, 85]. The advantages are as follows. Individuals diagnosed with psychiatric disorders are considered to have abnormal social relationships partly because they are a minority within the population, and do not fit into a society where the majority of (normal) individuals lack phenotypes associated with disorders. A similar relationship can be applied to domestic and wild animals. In particular, the social behavior of dogs is quite distinct from that of wild animals. Such social behavior in dogs that appear to have evolved as human companions may be considered as "abnormal" behaviors from the viewpoint of wild animals.

Dogs also vary in their neotenic features. Some breeds such as huskies and corgis in adulthood have a less neotenic appearance and look closer to the wild-types such as jackals and coyotes, whereas other breeds such as Saint Bernards and Great Pyrenees exhibit a stronger neotenic appearance, maintaining a juvenile-like appearance as adults[86]. In particular, neotenic appearance and cerebral DA level are correlated in dogs[86, 87]. Thus, breeds close to the wild-type and displaying a less neotenic appearance have higher cerebral DA levels than those with a more neotenic appearance[86, 87], further supporting our hypothesis of a relationship between lower DA levels and neoteny. Another advantage of using dogs for investigation is that they have a clear phylogeny, which enables investigators to follow their evolution relatively easily.

It is also interesting to note that unlike in humans, spontaneously-occurring neurological disorders such as Alzheimer's disease or Parkinson's disease are extremely rare, if they occur at all, in wild animals including non-human primates without artificial genetic or pharmacological manipulation. Dogs are an exception. They show spontaneously and endogenously-occurring Alzheimer's disease-like alterations in their brains[88] which, although not necessarily related to neotenic development of the PFC with DA signaling, illustrate that dogs have acquired a brain system that is closer to that of humans than other wild animals through evolution by domestication. Therefore, dogs could be a good model to understand how evolutionary change pertains to the emergence of human-specific brain disorders[89].

Conclusions

We have proposed a hypothesis that mesocortical DA plays a specific role in the development and evolution of the PFC. However, our hypothesis has several limitations. For instance, DA transmission in the PFC is regulated by brain areas such as limbic structures[90] and diencephalic nuclei including the habenula[91]. These structures and other parts

of the brain that regulate PFC DA release are relatively conserved across species (i.e. evolutionarily old brain areas), suggesting that, although this possibility cannot be excluded, decreased DA levels in the PFC may not be a consequence of altered regulatory mechanisms by other brain structures, but is more likely the result of an intrinsic change within the PFC selected through evolution. In addition, since our hypothesis is based on disorder models such as ADHD, schizophrenia, and chronic stress, it may be that these disorders involve brain mechanisms other than DA and PFC. However, this problem may be partly overcome by using multiple disease models. Thus, each disorder involves various mechanisms, and the differences between these mechanisms may create different conditions in the disorders, although one mechanism is common across the disorders (e.g. ADHD involves mechanisms A, B, and C, whereas schizophrenia involves mechanisms A, D, and E, and chronic stress involves mechanisms A, C, and F...). If the argument (i.e. role of DA in PFC development) is supported by all of these different conditions of disorders, then, the mechanism in this argument is most likely the one that is common across the disorders (i.e. DA change in the PFC), but not other mechanisms.

In conclusion, an evolutionary perspective for understanding the role of neurochemicals such as DA and their relation to brain disorders may open a new venue in neuroscience.

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·Review·

Prefrontal cortex and sensory cortices during working memory: quantity and quality

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The activity in sensory cortices and the prefrontal cortex (PFC) throughout the delay interval of working memory (WM) tasks reflect two aspects of WM—quality and quantity, respectively. The delay activity in sensory cortices is fine-tuned to sensory information and forms the neural basis of the precision of WM storage, while the delay activity in the PFC appears to represent behavioral goals and filters out irrelevant distractions, forming the neural basis of the quantity of task-relevant information in WM. The PFC and sensory cortices interact through different frequency bands of neuronal oscillation (theta, alpha, and gamma) to fulfill goal-directed behaviors.

Keywords: working memory; prefrontal cortex; capacity limit; memory precision; neuronal oscillations

Introduction

Working memory (WM) refers to the cognitive processes of maintaining and storing information in the short term (usually seconds) for subsequent goal-directed action[1-3]. Persistent activity in both sensory cortices and association areas (especially the prefrontal cortex, PFC) throughout the delay interval of a WM task after sensory stimulus presentation (sample) are usually considered to be critical for WM maintenance, and to bridge the temporal gap between the sample and the subsequent contingent response (see reviews [4,5]). However, with regard to WM the role of the delay activity in sensory cortices has been thought to differ from that of the PFC. The former has been thought to represent and store selective sensory information and the latter has been considered to exert attentional bias and cognitive control over the former (see reviews [6,7]).

Despite the vast storage in human long-term memory, WM has been demonstrated to have a capacity limited by the number of items[8,9] and this is strongly correlated with general cognitive ability[10,11]. Recent advances in studying visual WM have shown a precision limit of representations in WM besides the capacity limit[12-16].

Combined with the above findings, we propose that delay activity in the sensory cortices and PFC reflect the quality and quantity of representations in WM, respectively. Specifically, in this review, quantity refers to how many items/slots are stored in working memory, and quality refers to how precisely the features of each item/slot are represented in WM.

Sensory Cortices and the Quality of Working Memory

Neurons in the PFC have been shown to respond to sensory stimuli in WM tasks[17,18]. Compared with those PFC neurons, neurons in sensory cortices appear to be more selectively tuned to stimulus features in WM tasks

and consequently to maintain high-fidelity representations of stimulus information in the service of WM[19].

Some human imaging as well as neurophysiological studies in non-human primates have indicated an absence of persistent activity in early sensory regions[20-24]. However, other primate studies have revealed persistent modulation of neuronal activity in the primary visual cortex during the delay period of a WM task, which additionally correlates with the monkeys' memory performance[25]. Furthermore, Zhou and Fuster found that single units in monkey primary somatosensory cortex (SI) show sustained firing during the retention period of a tactile WM task[26] (Fig. 1A).

Recent advances in neuroimaging have shown the possibility of accurately decoding the representations of minds (see reviews [27,28]). Multivariate pattern analysis (MVPA) has been applied to the analysis of neural activity patterns in visual regions, and has revealed content-specific representations during WM[29,30]. Harrison and Tong have shown that even if the overall delay activity is low in human visual cortices, orientations held in WM can still be clearly decoded from the activity patterns[29]. This coincides with the neurophysiological data from monkey SI cortex during the delay period of a tactile WM task noted above[26]. Furthermore, recent neuroimaging studies have shown that trial-specific stimulus information can be decoded from sensory cortices but not from the PFC[31-33].

Not only do sensory cortices represent fine-tuned modality-specific sensory information during WM, but they can also be tuned to other sensory modalities (cross-modality) after associative training. Zhou and Fuster have shown that the sustained delay activity of SI neurons in monkeys is selective for visual stimuli in a visual-tactile cross-modal WM task[34] (Fig. 1B). Applying to humans a paradigm similar to that used in monkeys, Ku and colleagues have found that the source of delay activity localized in human SI is modulated by cross-modal associations at the early stage of the delay (100–200 ms after the onset of sample stimuli) and the modulation exhibits a bottom-up pattern[35]. Our recent transcranial magnetic stimulation (TMS) study has further shown that SI plays a causal role in performance in tactile-visual cross-modal WM[36] (Fig. 1C). Interestingly, using MVPA, Christophel and Haynes have decoded motion patterns not only from visual areas, but also from SI, even if the task is a pure visual WM task and the visual-spatial pattern is without any association with touch[37]. They have suggested that the tactile cross-modal representations are specific to complex dynamic stimuli[37].

As the contents of WM can be decoded from sensory cortices but not the PFC[31-33], we propose here that, compared with the PFC, sensory cortices represent more precise information about the memorandum, and in this way serve as quality assurance in WM.

Prefrontal Cortex and WM Quantity

Compared with relatively few studies on sustained delay activity in sensory cortices in WM tasks, the elevated delay activity in the PFC has long been recognized[38]. At first, this PFC activity was interpreted as encoding the sensory features of WM items[3]. However, growing evidence places more emphasis on is role in providing top-down control over the more posterior regions where information is primarily stored[39,40].

In psychological studies, the quantity limit of WM for human has long been characterized as approximately 7 verbal items[8] or 4 visual items[9]. This limit has been attributed to activity in parietal areas[41-43]. However, further studies have shown that high-capacity individuals are more efficient at filtering out irrelevant items, while low-capacity individuals cannot efficiently filter out such distractions[44]. This filtering ability seems to be particularly critical since the high- and low-capacity groups tend to have similar capacity limits counting the number of both targets and distractors[44]. The PFC has been shown to control accesses to WM[45,46] and can then guarantee the quantity of task-relevant items in WM.

A similar quantity limitation has also been revealed in monkey neurophysiological studies[46,47]. In addition, studies have shown that neurons in the PFC of numerically naive monkeys tune to a preferred numerosity[48], independent of sensory modality[49]. These neurons may potentially subserve the neuronal mechanisms underlying WM quantity.

The persistent delay activity in the PFC during WM has been demonstrated to be critical for maintaining behavioral goals and the means to achieve those goals (see review [6]). The number of goals simultaneously maintained, considering a goal/rule as an item/slot, can be regarded as the other aspect of WM quantity, the capacity of the central executive. This format of quantity is even more

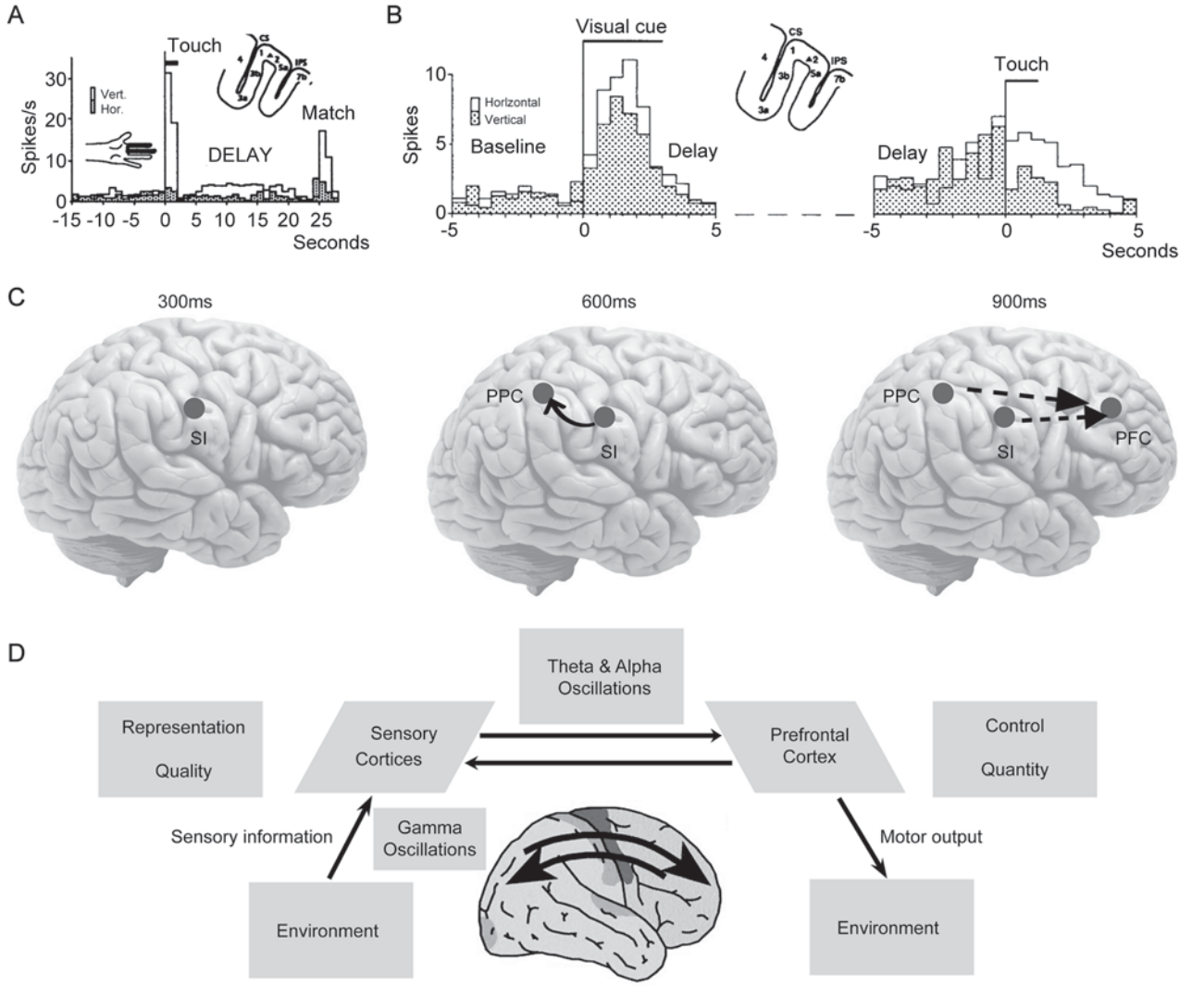

Fig. 1. (A) A primary somatosensory (SI) unit is activated differentially by touch and retention of the vertical edges (the sample stimulus). The receptive field of the unit is indicated in a diagram of the monkey's hand, and the location of the unit is marked by a triangle in a brain section diagram. In the study, the tactile stimuli used in a delayed matching-to-sample task are a pair of objects that differ in the direction of edges (vertical *versus* horizontal) on their surface (modified from [20]). (B) An SI unit favors the horizontal visual cue in the cue period as well as the horizontal ridges in the tactile choice. A pair of icons is used as visual cues in a visual-tactile cross-modal working memory (WM) task; they are black-and-white patterns of parallel stripes, vertical in one icon and horizontal in the other (modified from [25]). (C) A possible model of cross-modal WM proposed in a transcranial magnetic stimulation study. The early storage of tactile information is processed and briefly maintained in the contralateral SI, and the information is later transferred to the posterior parietal cortex and PFC[36]. (D) Schematic showing the representation of sensory input in sensory cortices reflects the quality aspect of WM, and the prefrontal top-down control activity reflects the quantity aspect of WM. The sensory cortices are synchronized in gamma cycles, and interact with the PFC to fulfill the needs of goal-directed behavior. Theta and alpha oscillations serve the interaction between sensory cortices and the PFC.

severely limited. Charron and Koechlin have proposed that the frontal lobes in the two hemispheres represent two concurrent goals[50]. Similar hemispheric limitation has also been proposed through neurophysiological data from non-human primates[47]. Further, the frontal lobe in each hemisphere can be subdivided according to the abstraction

of processed goals, which may result in different capacity limits for each hierarchy[51]. Although the quantity of the capacity limitation for the central executive in WM is still not well-defined, as pointed out above, the quantity property of WM does exist in the PFC.

Neural Oscillations Serve the Interaction between PFC and Sensory Cortices

A complete WM function requires the combination of quantity and quality, which relies on the coordination of the PFC and sensory cortices. It has been suggested that oscillatory synchronization underlies inter-cortical communications[52]. In this review, we mainly focus on three frequency bands that are important to WM performance in humans as well as non-human primates: theta (4-8 Hz), alpha (8–13 Hz), and gamma (>30 Hz).

Cortical theta rhythms are probably generated in hippocampal-cortical feedback loops[53]. Frontal theta occurs during WM in both humans and non-human primates[46,53-55]. The theta power increases during WM encoding, maintenance, and retrieval, compared with its baseline level[56]. Theta power is modulated proportionally to the number of memoranda[57,58], which represents the quantity of WM. Long-range theta coupling between the PFC and sensory cortices serves communication between these areas and can also influence behavior, as it has been shown that theta coupling between V4 (sensory association cortex) and the PFC predicts WM performance[59].

Alpha oscillations have been known to be the most dominant rhythm in scalp electroencephalography since its discovery almost a century ago[60]. The alpha rhythm was originally associated with an idling mental state, but has recently been found to play a functional inhibitory role in attention and WM (see review [61]). The cortical alpha rhythm is thought to be generated *via* thalamocortical and cortico-cortical loops[62]. It has also been shown to increase with the number of items to be remembered in different sensory domains[63,64], and might represent the quantity of WM. Jensen and colleagues have proposed a model that the magnitude of alpha oscillations actually determines how many representations are processed[65]. However, the oscillations may mainly serve to protect the information of WM memoranda from distractions[66,67], as alpha power also goes up when the number of distractors increases[68]. A recent TMS study has shown that frontal-parietal alpha oscillations can be modulated by PFC activity[69], which implies that alpha oscillations could also be a working band for communication between the PFC and sensory cortices during WM. Besides the above studies linking alpha oscillations with WM quantity, two new studies have indicated their role in WM precision[70,71], so it cannot be ruled out that alpha oscillations are also correlated with WM quality. Future studies to disentangle the roles of alpha power and phase would help to answer this question, since it has been shown that the phase-locked and non-phase-locked parts of alpha oscillations are related to different processes during WM[72].

Gamma synchronization was first found to subserve perceptual binding[73,74]. Recently, this synchronization has been suggested to be critical to WM (see reviews[75,76]). Although there is no direct evidence linking gamma oscillations to WM precision, their functional role in mentally representing objects[77] and predicting successful memory encoding[78] has led us to propose a role of gamma oscillations in WM quality. It should also be noted that a large number of studies link WM load to the amplitude of gamma oscillations[79-82]. However, as the number of memoranda (WM load) increases, more feature information regarding the memoranda needs to be remembered to successfully perform WM tasks. In addition, gamma activity has been suggested to be more important in sensory binding or even in multisensory integration[83]. It therefore seems that it is a more plausible assumption to connect gamma oscillations to their role in WM quality. Future studies to disentangle the contribution of the number of items/slots or features to the modulation of gamma oscillations will be of interest.

Taken together, these works suggest that WM quality is likely maintained in higher-frequency oscillations, as in the gamma cycle, or to some extent in the alpha band, and WM quantity is related to lower-frequency oscillations, such as theta and alpha. In a recent review, Roux and Uhlhaas have proposed that the cross-frequency coupling of theta-gamma or alpha-gamma codes for distinct WM information: sequentially verbal or visuo-spatial information, respectively[84]. Therefore, gamma activity likely represents objects in certain phases of theta and alpha activity, in which the PFC and sensory cortices communicate with

each other to accomplish WM performance.

Some Topics Not Completely Covered in This Review

Given space limitations, we could not cover every aspect of the topic of quality and quantity in WM. However, several important issues merit brief consideration.

The posterior parietal cortex (PPC) also plays an important role in WM and is critical for the capacity limit[43]. Recent studies have suggested that inferior and superior portions of the PPC represent different types of WM information during the delay period, the inferior portion indicating the binding between spatial locations and memoranda, and the superior portion specifying the complexity of sample stimuli[41,85,86]. Therefore, the PPC may be involved in both the quantity and quality aspects of WM.

Besides the roles of theta, alpha, and gamma oscillations in WM, beta oscillations (20–30 Hz) recorded at frontal sites have also been shown to be parametrically modulated in WM by sensory stimuli[64,67,87-89]. Therefore, the beta rhythm might be another candidate for representing WM quality.

Drawn from a plethora of studies, we propose here that the WM quality is represented in sensory cortices and the WM quantity is represented in the PFC. However, the opposite cannot be ruled out. It is notable that the quality of representation decoded from sensory cortices declines with increasing quantity of memoranda[32]. On the other hand, as suggested above, frontal beta oscillations represent parametric sensory information[64,67,87-89]. Future work on the bi-directional influence between quality and quantity would be helpful to disentangle these intertwined factors.

Concluding Remarks

Here, we propose a framework with the fine-tuned sensory representation in sensory cortices, which reflects the quality aspect of WM and is carried on by higher-frequency neural oscillations (gamma, beta/alpha), and the prefrontal top-down control activity that reflects the quantity aspect of WM and is carried on by lower-frequency neural oscillations (theta/alpha). As quality and quantity are intertwined and essential parts of WM, activity in the sensory cortices and PFC during WM interacts to fulfill the requirements of goal-directed behavior, and higher- *versus* lower-frequency oscillations might serve as the communication frequency to synchronize both intra- and inter-area activities (Fig. 1D). Future work to assess neuronal activity simultaneously in both the PFC and sensory cortices in WM tasks will be of great interest.

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·Review·

Dopaminergic modulation of synaptic plasticity in rat prefrontal neurons

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The prefrontal cortex (PFC) is thought to store the traces for a type of long-term memory – the abstract memory that determines the temporal structure of behavior often termed a “rule” or “strategy”. Long-term synaptic plasticity might serve as an underlying cellular mechanism for this type of memory. We therefore studied the induction of synaptic plasticity in rat PFC neurons, maintained *in vitro*, with special emphasis on the functionally important neuromodulator dopamine. First, the induction of long-term potentiation (LTP) was facilitated in the presence of tonic/background dopamine in the bath, and the dose-dependency of this background dopamine followed an “inverted-U” function, where too high or too low dopamine levels could not facilitate LTP. Second, the induction of long-term depression (LTD) by low-frequency stimuli appeared to be independent of background dopamine, but required endogenous, phasically-released dopamine during the stimuli. Blockade of dopamine receptors during the stimuli and exaggeration of the effect of this endogenously-released dopamine by inhibition of dopamine transporter activity both blocked LTD. Thus, LTD induction also followed an inverted-U function in its dopamine-dependency. We conclude that PFC synaptic plasticity is powerfully modulated by dopamine through inverted-U-shaped dose-dependency.

Keywords: prefrontal cortex; synaptic plasticity; long-term memory

Introduction to Synaptic Plasticity and the Prefrontal Cortex

The prefrontal cortex (PFC), the cortical area most developed in humans, is known to serve for higher cognitive or executive functions[1]. Deficits in the PFC are thought to underlie the cognitive disturbances seen in psychiatric disorders including schizophrenia, depression, drug addiction, and attention deficit and hyperactivity disorder[2]. Therefore, understanding the physiological and pathophysiological bases of PFC neuronal function helps to understand the cellular basis of higher cognitive abilities and their disturbance. In this regard, it should be noted that the PFC receives innervation from dopaminergic fibers from the ventral tegmental area[3], and that rat PFC neurons express dopamine (DA) receptors in different layers[4]. This dopaminergic projection is important for diverse cognitive functions including working memory, goal-direction, and other executive functions[5,6].

Working memory has attracted much attention as a major example of PFC executive functions. Indeed, revealing the cellular basis of working memory is a major accomplishment of neuroscience research[7]. The reasons that working memory research attracts attention are that working memory is clearly a critical component of higher mental activity and that its impairment is a core feature of the cognitive symptoms of schizophrenia[8].

It is well known that working memory is the short-term maintenance of a retrieved/acquired memory about facts and objects. Given the tight association between this short-

term memory and PFC function, the role of the PFC in long-term memory has often been overlooked. But it is clear that the functions of the PFC include a long-term memory component[1], and the cognitive symptoms of schizophrenia also include deficits in long-term memory[9].

The long-term memory we suggest to be attributable to the PFC is a higher-order, abstract memory labelled as "temporal organization of behavior", "rule", "strategy", or "planning"; that is, a memory that determines the sequence of outputs of concrete actions/ideas[2]. We propose that the traces for this type of long-term memory are at least partly stored in neurons of the PFC in the form of long-term synaptic plasticity[2]. Indeed, injection of a protein synthesis inhibitor known to block the maintenance of synaptic plasticity into the prelimbic area (rodent PFC) severely impairs the acquisition of goal-directed action sequences[10].

Based on this important relationship between long-term memory and the PFC, here we review long-term synaptic plasticity, i.e. long-term potentiation (LTP) and long-term depression (LTD), two of the cellular mechanisms for memory encoding and storage, in rodent PFC neurons (the prelimbic area). Our particular interest is the induction mechanisms of LTP and LTD with special emphasis on DA, an important neuromodulator of PFC function.

LTP in PFC Neurons and Dopamine

LTP and Background Dopamine

LTP is typically induced by trains of high-frequency stimuli delivered to presynaptic fibers. So, we applied stimuli at 50 Hz (4 or 6 trains of 100 pulses, delivered at 10-s intervals) to layer I-II presynaptic fibers in rat PFC slices, and monitored the changes in the excitatory postsynaptic potentials (EPSPs) recorded from layer V pyramidal neurons. The frequency 50 Hz is within the range of functionally important γ-band activity in the PFC[11]. In addition, all main experiments were conducted in the presence of the GABA-A receptor antagonist bicuculline so that inhibitory postsynaptic potentials were largely eliminated.

We found that while hippocampal neurons readily show LTP after such 50-Hz stimulation, in PFC neurons, the stimuli either induce no plasticity (trains delivered 4 times; Fig. 1A) or induce LTD (delivered 6 times; Fig. 1B). These results are in contrast to the result obtained in the rat PFC *in vivo*, where high-frequency afferent stimuli always induce LTP[12].

An important difference between *in vivo* and *in vitro* preparations is that in the latter, the extracellular DA level is very low, perhaps to a degree that is non-physiological. In the PFC *in vivo*, in contrast, background DA is maintained even under anesthesia by the tonic spontaneous activity of midbrain dopaminergic neurons[13]. Therefore, in an attempt to mimic the *in vivo* condition in the slice preparation, we added to the bathing medium a low concentration of DA (3 μmol/L) as background, continuously for 40 min before the delivery of the identical 50-Hz stimulation. Under this condition, the delivery of trains 4 and 6 times both induced clear LTP (Fig 1C, D)[14,15]. Thus, the background DA secured the induction of LTP.

The concentration of 3 μmol/L was chosen because it is about the lowest concentration of exogenous DA that elicits detectable changes in the EPSP under our recording condition (i.e. slight reductions or slight augmentations of the responses). In behaving animals, however, the DA concentration, in the nucleus accumbens for example, estimated by voltammetry is ~1 μmol/L or less, even at the peak[16]. Compared with this, 3 μmol/L may appear high. However, the probe used in the voltammetry has a diameter of ≤10 μm, which is as large as the diameter of a cell body. The real concentration near a synapse is likely to be much higher, possibly reaching the mmol/L range[17].

Constraints on LTP Facilitation by Background Dopamine

We discovered two functionally important constraints in the LTP facilitation by background DA described above. First, the level of the background DA has to be within a certain range: too high (10 μmol/L) or too low (1 μmol/L) a concentration does not facilitate LTP[17]. This finding indicates that the dose-dependency of LTP induction on the background DA follows an inverted-U dose-response curve, reminiscent of the relationship between the level of DA in the PFC and its modulatory action on PFC-dependent cognitive functions[2, 6]. Second, the background DA, even at an appropriate level (3 μmol/L in our case), has to be present long enough to facilitate LTP. Thus, the 3 μmol/L DA facilitated LTP after 40 min bath-application, but not after 12.5 min[14]. This is reminiscent of the fact that background DA is continuously present in the PFC *in vivo*. In addition,

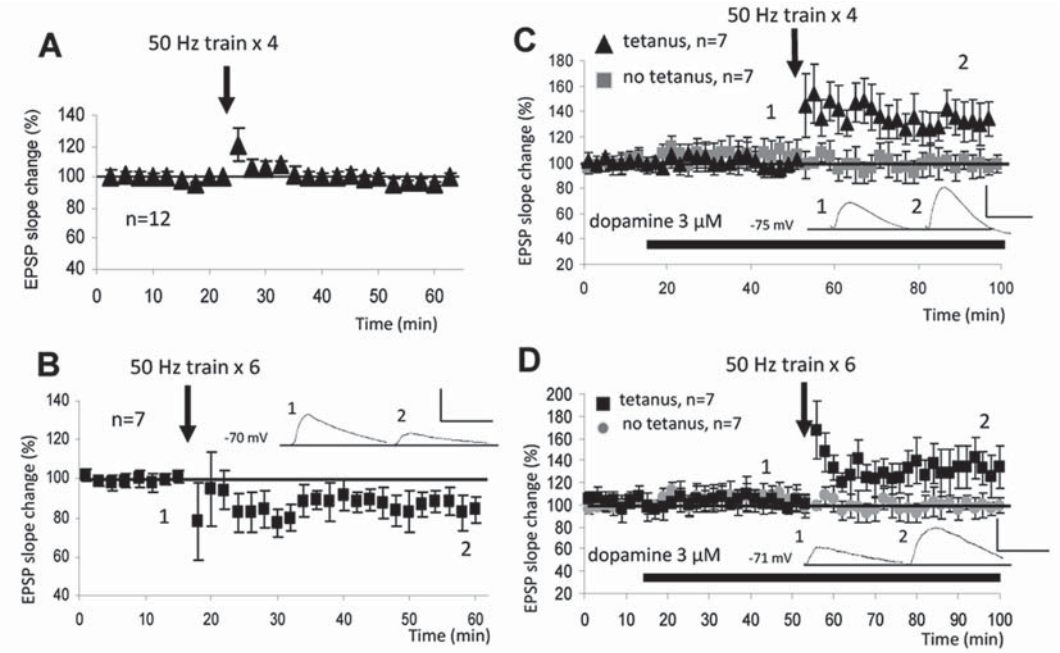

Fig. 1. Effect of high-frequency stimuli on the EPSP recorded from layer V pyramidal neurons in rat prelimbic cortex slices. A. Delivery of 50-Hz stimuli (100 pulses, 4 trains at 10-sec intervals) to layer I-II presynaptic fibers in prelimbic slices induces no lasting changes in the EPSP. B. The stimuli were delivered 6 times. In this case, clear LTD is induced. The insets are the averaged EPSP recorded just before (1) and 40 min after stimulation (2). C. Delivery of 50-Hz stimuli 4 times (as in A) after 40 min perfusion with 3 µmol/L background dopamine results in LTP (black triangles). The insets are the averaged EPSP recorded just before (1) and 40 min after stimulation (2). The 3-µmol/L background dopamine itself does not modify the EPSP (grey squares). D. Delivery of 50-Hz stimuli 6 times (as in B) after 40 min perfusion with 3 µmol/L background dopamine converts the LTD to LTP (black squares). The insets are the averaged EPSP recorded just before (1) and 40 min after stimulation (2). The 3-µmol/L background dopamine itself does not modify the EPSP (grey circles). Scales in B–D: vertical 10 mV, horizontal 50 ms. Adapted from Kolomiets *et al.* (2009)[15], with permission.

DA acts on both D1 and D2 receptors to facilitate LTP[15].

Mechanisms Underlying the Action of Background Dopamine

The above results indicate that LTP is induced in the PFC only when the physiological conditions are mimicked: thus, an appropriate level of background DA (3 µmol/L in our case) must be present for a certain time (40 min in our case) in order to successfully facilitate LTP.

What, then, is the molecular mechanism underlying this DA effect? Our analysis indicated that it is the activation of extracellular signal-regulated kinases (ERK1/2). We first confirmed that LTP requires the postsynaptic activation of ERK[15]. We then quantified the phosphorylated ERK level in the PFC by western blot analysis and found that ERK phosphorylation increases slowly in the presence of 3 µmol/L DA[15]. More precisely, a significant increase in the phosphorylated ERK occurs after 40 min perfusion (LTP condition) but not after 12.5 min perfusion (non-LTP condition). Equally, in two other non-LTP conditions (40 min perfusion of 1 or 10 µmol/L DA) no increased ERK phosphorylation is seen. Moreover, under the condition where the increased ERK phosphorylation by 40-min perfusion of 3 µmol/L DA is lowered to the control level by a brief bath-application of ERK inhibitor PD98059, LTP induction is also blocked, suggesting a causal relation between the slow increase of ERK phosphorylation and LTP induction.

Apart from the above inverted-U dose-response activation of ERK, however, little is known as to how the background DA regulates LTP through the inverted U-fashion. For example, background DA does not affect N-methyl-D-aspartate receptor-mediated synaptic transmission[15], unlike the previous report[18]. One candidate for the underlying mechanism is a dose-dependent, D1-

mediated increase of neuronal excitability[19]. Indeed, background DA does enhance the postsynaptic depolarization during LTP-inducing high-frequency input[15]. However, this enhancement does not follow an inverted-U curve, since 1 or 10 μmol/L DA, which does not facilitate LTP, still enhances the depolarization[15]. Also, unlike the report by Chen *et al*.[19], this enhanced postsynaptic depolarization persists in the presence of the ERK inhibitor PD98059[15]. Thus, while it is likely that the enhanced postsynaptic depolarization by background DA contributes to the induction of DA-facilitated LTP, other cellular processes must co-exist to realize the inverted-U, dose-dependent regulation of LTP.

LTP and Phasic Dopamine

As well as the tonic/background DA, which is maintained by the spontaneous, basal firing of DA neurons, event-related phasic release of DA, which is correlated with a transient, event-related high-frequency discharge of DA neurons *in vivo*, is functionally important[20]. This phasic DA, when occurring in temporal conjunction with glutamatergic synaptic activity, facilitates LTP in the PFC *in vivo*[12]. In our case, equally, the same conditioning stimuli activated both glutamatergic and dopaminergic axon terminals in PFC slices. The functional importance of this timing between dopaminergic and glutamatergic inputs has also been shown in a recent report by Yagishita *et al*.[21] in striatal neurons. We thus also examined how phasic DA is involved in LTP induction *in vitro*.

First, blockade of D1 or D2 receptors by the specific antagonist SCH23390 (2 μmol/L) or sulpiride (20 μmol/L), respectively, only during the delivery of 50-Hz stimulation, reliably blocks LTP in the presence of 3 μmol/L background DA (Fig. 2A). This indicates that endogenous, stimulus-evoked phasic release of DA is required for LTP (note that there is a remote possibility that the brief absence of tonic DA action while the antagonists are present causes the LTP blockade). In PFC slices, the axons of dopaminergic neurons are severed, but the residual axon terminals release DA upon repetitive stimulation and induce plasticity[22]. Indeed, the superficial layers of the rat PFC receive dopaminergic innervation[23], and rat frontal pyramidal neuron dendrites co-express D1 and D2 receptors[4]. Second, the LTD induced by delivery of 50-Hz stimulation 6 times in the absence of background DA (see Fig. 1B) is also blocked by SCH23390 or sulpiride (Fig. 2B), indicating that this LTD also depends on phasic endogenous DA. Note that in Fig. 2B, a small LTP appears when either D1 or D2 receptors are blocked. Such LTP does not occur when D1 and D2 receptors are simultaneously blocked[15]. This indicates that without background DA (an abnormal condition) an imbalance of stimulation between these two receptor classes can give rise to response potentiation through as yet unknown mechanisms.

Thus, the phasic DA released upon 50-Hz stimulation can induce either LTP or LTD through the co-activation of D1 and D2 receptors, which might result in the synergistic

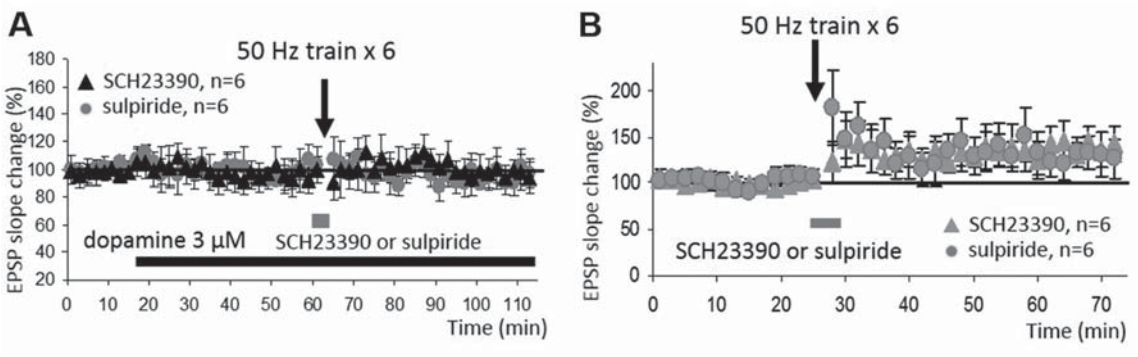

Fig. 2. Induction of LTP and LTD by 50-Hz stimulation requires phasic dopamine release. A. Brief bath-application of the D1 receptor antagonist SCH23390 (2 μmol/L; black triangles) or the D2 receptor antagonist sulpiride (20 μmol/L; grey circles) during LTP-inducing 50-Hz stimulation (6 times) after 40 min perfusion with 3 μmol/L background dopamine (see Fig. 1D) blocks the induction of LTP. B. The identical application of SCH23390 or sulpiride as in A during LTD-inducing stimulation (delivery of 50-Hz stimulus train 6 times without background dopamine) blocks the induction of LTD. Adapted from Kolomiets *et al.* (2009)[15] with permission.

activation of phospholipase C[4]. These opposite effects of DA suggest that its phasic release serves as a "trigger" for plasticity but it does not determine the direction of the plasticity (i.e. LTP or LTD); the direction of plasticity is determined by the level of background DA. Thus, when the level of background DA is appropriate (3 μmol/L in our case), the phasic DA triggers LTP (Fig 1C, D); but when the level is low (1 μmol/L) or high (10 μmol/L), the phasic DA cannot trigger LTP. When the level is extremely low (absence of background DA in our case; see Fig. 1B), the same phasic DA now triggers LTD. Our additional data[14,24,25] suggest that when the level of background DA is extremely high (100 μmol/L), the phasic DA also triggers LTD. These relationships between phasic and background DA in terms of plasticity induction are graphically presented in Figure 3.

LTD in PFC Neurons and Dopamine

Induction of LTD by Low-Frequency Repetitive Stimuli

In the PFC, the induction of LTD by low-frequency repetitive stimulation has been demonstrated in mouse brain slices[26]

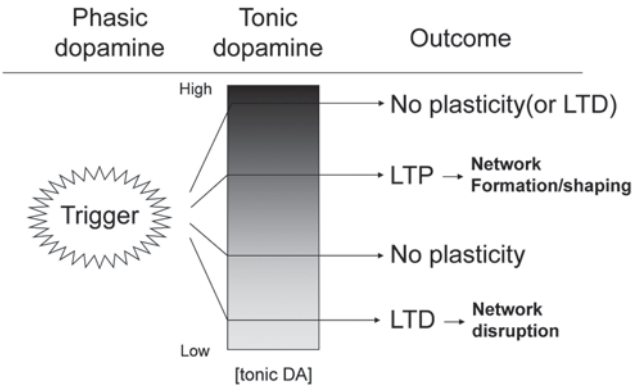

Fig. 3. Schematic representation of the relation between phasically-released dopamine upon high-frequency input and tonic/background dopamine in terms of plasticity induction in the PFC. The stimulus-evoked phasic dopamine serves as a "trigger" for plasticity, but does not determine the direction of plasticity. The direction (i.e. potentiation or depression) is determined by the level of tonic/background dopamine. The phasic dopamine only triggers LTP at appropriate levels of tonic/background dopamine. Under very low or very high levels of tonic/background dopamine, the phasic dopamine triggers LTD, which we term as "aberrant LTD". Adapted from Goto *et al.* (2010)[2] with slight modifications.

but had never been shown in rat preparations. Therefore, we first determined whether low-frequency stimuli (3 Hz for 15 min)[26] delivered to layer I-II afferent fibers induces LTD in rat PFC slices. We found that the 3-Hz stimulation successfully induces LTD of the EPSP (Fig. 4A)[27], monitored in this case by an extracellular microelectrode in layers I-II[28].

This LTD by 3 Hz stimulation was induced even in the presence of 3 μmol/L background DA (data not shown). This is in sharp contrast to the LTD induced by 50 Hz, which converts to LTP when 3 μmol/L DA is added to the bath (Fig 1B, D). This difference may indicate that LTD induced by 3 Hz stimulation is a physiologically relevant form of synaptic depression that persists in the presence of background DA[27].

Inhibition of Dopamine Transporter Activity and LTD

LTD by 3 Hz stimuli is blocked by the D1 receptor antagonist SCH23390 (2 μmol/L) or the D2 receptor antagonist sulpiride (20 μmol/L) applied during the 3 Hz stimulation (Fig. 4B1 and 4B2). Thus, this LTD depends on endogenously-released DA acting on both D1 and D2 receptors; that is, levels of receptor activation by phasically-released DA that are too low are insufficient for LTD. Is this LTD then inhibited by levels of phasic DA that are too high, forming an inverted-U dose-response curve?

To test this possibility, we elevated the extracellular DA level by inhibiting the DA transporter (DAT) using selective blockers. Since the DAT is inhibited by cocaine, this study is also important with regard to the molecular mechanisms of drug addiction. Cocaine also inhibits the norepinephrine and serotonin transporters, but the reinstatement of cocaine addiction occurs specifically through the drug's action on the DAT in the PFC[29,30].

From the functional aspect, the involvement of DAT inhibition in the PFC in the reinstatement of cocaine addiction predicts that LTD in the PFC would be impaired by DAT inhibitors. This is because reinstatement is the condition where behavioral flexibility is diminished by re-exposure to cocaine so that the individual becomes unable to suppress the old goal-direction, i.e. cocaine-seeking. Since the main function of LTD in the PFC is to guarantee behavioral flexibility by suppressing old goal-directions[31], the inhibition of DAT in the PFC, which diminishes behavioral flexibility, should inhibit LTD.

As predicted, the highly-selective DAT inhibitor GBR12909 (1–200 nmol/L) or GBR12935 (100 nmol/L),

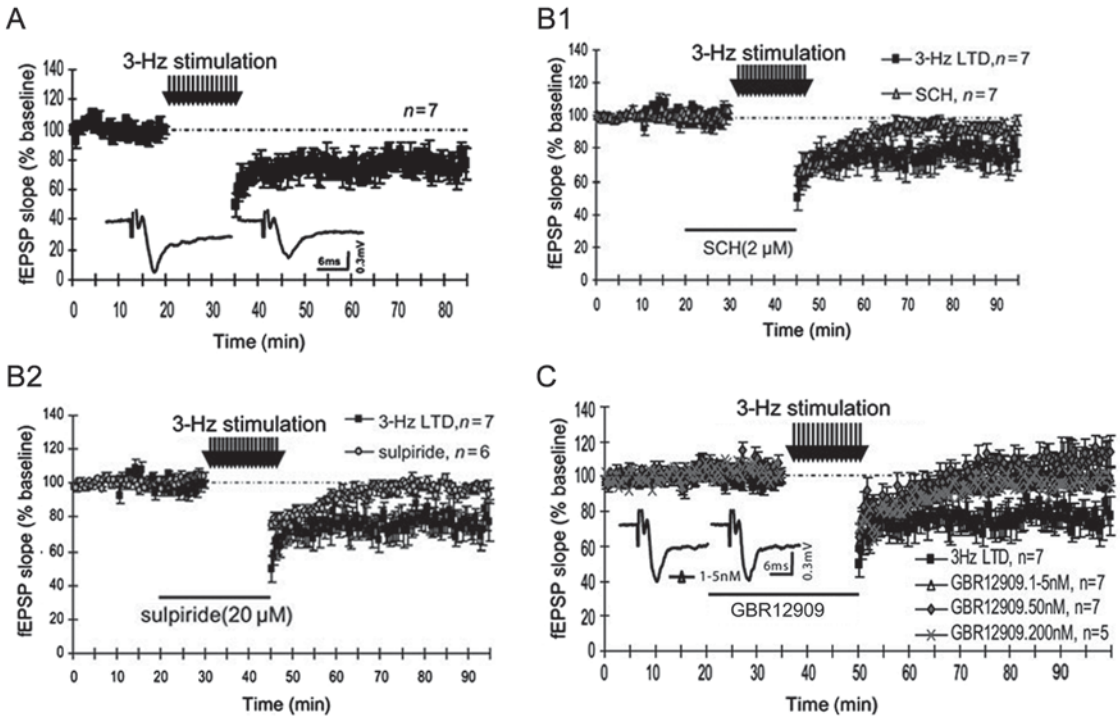

Fig. 4. Blockade of dopamine transporter activity impairs LTD induced by low-frequency repetitive stimulation. A. Delivery of single stimuli at 3 Hz (for 15 min) to layer I-II presynaptic fibers induces stable LTD of the EPSP recorded extracellularly from layers I-II. The insets are the averaged EPSPs recorded just before (left) and 40 min after stimulation. Scales: vertical, 0.3 mV; horizontal, 6 ms. B1. LTD induced by 3 Hz stimuli is blocked by the D1 antagonist SCH23390 (2 μmol/L) applied during stimulation (grey triangles). B2. LTD induced at 3 Hz is blocked by the D2 antagonist sulpiride (20 μmol/L) applied during stimulation (grey circles). C. Augmentation of the extracellular dopamine level by bath-application of the DAT inhibitor GBR12909 (white triangles, 1–5 nmol/L; white diamonds, 50 nmol/L; crosses, 200 nmol/L) during LTD-inducing 3-Hz stimulation blocks the induction of LTD. The insets are averaged EPSPs recorded just before (left) and 40 min after stimulation (right) in the 1–5 nmol/L group. Adapted from Bai *et al.* (2014)[27] with permission.

bath-applied during 3-Hz stimulation, significantly impairs LTD (Fig. 4C)[27]. This impairment appears to be due to an over-stimulation of D1 receptors, since counteracting D1 receptor stimulation by co-applying a low level of SCH23390 (1 μmol/L) together with GBR12909 cancels the blocking action of GBR12909 on LTD[27]. Such blockade is not seen when a low level of sulpiride (10 μmol/L) is co-applied with GBR12909. Since 10 μmol/L sulpiride itself is insufficient to block LTD[27], the persistent blockade of LTD with sulpiride + GBR12909 is not because the sulpiride blocked LTD; rather, it is likely that the D2 antagonist sulpiride does not counteract the LTD-blocking action of GBR12909[27].

Taken together, these results indicate that DA controls LTD induction also through an inverted-U dose-response manner. This dependency on DA appears to be critically determined by the level of D1 receptor stimulation.

Molecular Mechanism of LTD Impairment by Dopamine Transporter Inhibition

What is the molecular mechanism of LTD impairment by DAT inhibition? According to our western blot analysis, it involves over-activation of ERK1/2, a class of messengers also necessary for LTD by DA[27]. First, ERK activity in the prelimbic area significantly increases in the LTD-impaired condition, i.e. 3-Hz stimulation in the presence of GBR12909. This ERK increase is not seen when LTD impairment is blocked, i.e. when SCH23390 (1 μmol/L) is co-present with GBR12909. Second, when the ERK increase seen with 3-Hz stimulation + GBR12909 is counteracted by simultaneous application of a low concentration of the ERK inhibitor PD98059 (5 μmol/L; 1/10 of the IC_{50} value[32]), the

LTD blockade by GBR12909 is also counteracted. Third, the allosteric positive modulator of mGluR5 (metabotropic glutamate receptor 5), CDPPB (3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide), which we found cancels the impairment of LTD by GBR12909, also cancels the over-activation of ERK by GBR12909.

Thus, we suggest that over-stimulation of DA (D1) receptors during GBR12909 application leads to over-activation of ERK1/2, which results in LTD impairment. The detailed molecular mechanism as to how the ERK over-activation occurs and how it impairs LTD remains to be clarified. It is also still unknown how CDPPB downregulates ERK activity. In addition, the over-activation of ERK1/2 under hyper-dopaminergic conditions seems inconsistent with our earlier data showing that 10 μmol/L background DA does not increase ERK1/2 activity, while 3 μmol/L does. This inconsistency is currently unexplained, although it may be related to the difference between the bath application of background DA and its stimulus-evoked endogenous release. Whatever the case, over-activation of ERK1/2 has also been shown with cocaine intake in rodents [33].

LTD and Background Dopamine

A major difference between LTP and LTD in our model system is that LTD can be induced even without background DA[27]. Functionally, this may indicate that the physiological role of LTD (suppression of old goal-directions[30]), persists even under extremely low levels of background DA. But under such a pathophysiological condition, LTP, unlike LTD, either cannot be induced (Fig. 1A) or converts to LTD (Fig. 1B). This latter LTD induced by LTP-inducing high-frequency stimulation under hypo-dopaminergic conditions should be termed “aberrant LTD” and separated from the physiological LTD induced by low-frequency stimulation. Aberrant LTD can be seen also with high-frequency stimulation in the presence of very high DA (100 μmol/L)[24,25]. Thus, when the concentration of background DA deviates greatly from the normal range, synaptic efficacy in the PFC neuronal network might be abnormally low.

Conclusion

Both LTP and LTD in rat PFC glutamatergic synapses show dependence on the DA level, characterized by the inverted-U shape function. LTP even converts to LTD if the background DA level is very low, as may occur in the PFC of chronically-stressed individuals or schizophrenic patients. Under these conditions, physiological LTD may still persist. As a result, synaptic efficacy in the PFC network might overly lower. The level of background DA may also be influenced by the emotional state, where acute aversive conditions appear to give rise to PFC extracellular DA levels more effectively than appetitive conditions[34-36]. Such an acute state may set the background DA at an optimal level to promote LTP, as shown in the hippocampus[37], and this may lead to better memory encoding as known empirically.

On the other hand, the psychoactive drug cocaine may exaggerate the action of stimulus-evoked, phasic DA release and impair physiological LTD. This action may lead to an impaired behavioral flexibility. The positive allosteric modulator of mGluR5 may serve as a treatment option for this cocaine-induced rigid goal-direction.

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·Review·

Contribution of NMDA receptors to dorsolateral prefrontal cortical networks in primates

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Cognitive disorders such as schizophrenia and Alzheimer's disease are associated with dysfunction of the highly evolved dorsolateral prefrontal cortex (dlPFC), and with changes in glutamatergic N-methyl-*D*-aspartate receptors (NMDARs). Recent research on the primate dlPFC discovered that the pyramidal cell circuits that generate the persistent firing underlying spatial working memory communicate through synapses on spines containing NMDARs with NR2B subunits (GluN2B) in the post-synaptic density. This contrasts with synapses in the hippocampus and primary visual cortex, where GluN2B receptors are both synaptic and extrasynaptic. Blockade of GluN2B in the dlPFC markedly reduces the persistent firing of the Delay cells needed for neuronal representations of visual space. Cholinergic stimulation of nicotinic α7 receptors within the glutamate synapse is necessary for NMDAR actions. In contrast, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors have only subtle effects on the persistent firing of Delay cells, but contribute substantially to the firing of Cue and Response cells. Systemic administration of the NMDAR antagonist ketamine reduces the persistent firing of Delay cells, but increases the firing of some Response cells. The reduction in persistent firing produced by ketamine may explain why this drug can mimic or worsen the cognitive symptoms of schizophrenia. Similar actions in the medial PFC circuits representing the emotional aspects of pain may contribute to the rapid analgesic and anti-depressant actions of ketamine.

Keywords: glutamate; Alzheimer's disease; schizophrenia; depression; ketamine

Introduction

Glutamate acts at a variety of ionotropic receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), kainate receptors, and N-methyl-*D*-aspartate receptors (NMDARs)[1]. NMDARs have been of particular interest due to their unique properties: they require depolarization to relieve their Mg^{++} block, and are permeable to Ca^{++} that can initiate second-messenger signaling events, such as mediating neuroplasticity or negative feedback through Ca^{++}-sensitive K^{+} channels. NMDARs contain an NR1 subunit and a mixture of NR2A–D subunits that alter the functional properties of the receptor, e.g. NMDARs with NR2A subunits (GluN2A) are more sensitive and have faster kinetics, while those with NR2B subunits (GluN2B) have slower kinetics and can produce increased levels of calcium influx[2]. As NMDARs are altered in cognitive disorders such as schizophrenia and Alzheimer's disease, there has been increasing research on these receptors[3, 4]. The highly-evolved dorsolateral prefrontal cortex (dlPFC) is only found in primates[5] and subserves higher cognitive functions, especially those affected in these mental disorders[6, 7]. The following review briefly summarizes new data demonstrating the key role of GluN2B receptors in the primate dlPFC, and how their actions in the dlPFC appear to differ from classical findings in the sensory cortex and hippocampus.

NMDAR and AMPAR Actions in Visual Cortex and Hippocampus

There have been extensive studies on the glutamate

NMDAR and AMPAR mechanisms underlying long-term synaptic plasticity in the primary visual cortex and in CA1 neurons of the hippocampus[8-10]. Long-term plasticity is powerfully regulated by the levels of AMPAR expression: the number of AMPARs inserted into the post-synaptic density can mediate the degree of spine depolarization and thus the NMDAR opening. AMPAR membrane insertion leads to structural synaptic changes such as enlarging the spine head and shortening/thickening of the spine neck[11, 12] to create a stable, mushroom-shaped spine and enduring strengthening of a synaptic connection[13], and/or the addition of new spines and synapses[11]. Synaptic plasticity in the mature visual cortex appears to be governed by GluN2A subunits, which have faster kinetics than GluN2B. GluN2B receptors are expressed in synapses early in development, but many move to extra-synaptic locations in the mature visual cortex and hippocampus[14]. In the hippocampus, there is some evidence that long-term potentiation (LTP) is mediated by synaptic GluN2A, while long-term depression is mediated by extrasynaptic GluN2B receptors[8]. However, this finding is controversial. For example, there is increasing evidence that GluN2B receptors are also important for LTP in hippocampal neurons[15-17]. In the mature visual cortex, long-term plastic changes in synapses appear to rely heavily on GluN2A, e.g. a selective GluN2A antagonist inhibits LTP induction, while neither a GluN2B antagonist[18] nor the over-expression of GluN2B[19] alters LTP in the visual cortex. The faster kinetics of GluN2A is well-suited to the rapid processing of continuous visual inputs and more faithful neuronal firing to sensory stimulation. Thus, it is appropriate that GluN2A actions predominate in visual cortical synapses.

NMDARs in Primate Dorsolateral Prefrontal Cortex

In contrast to sensory cortex, the dlPFC generates mental representations in the absence of sensory stimulation and these are the foundation of abstract thought. The dlPFC subserves working memory: the ability to keep information in mind and use these representations to provide top-down guidance of behavior, thought, and emotion. Working memory is active and relevant only for a short period of time, usually on the scale of seconds. This capability is a basic building block for more complex dlPFC cognitive operations. Working memory contrasts with long-term memory consolidation: it is a momentary, ever-changing pattern of recurrent activation of relatively stable architectural networks, while long-term memory consolidation retains events as structural changes in synapses. It is not surprising that the circuitry and modulation of working memory differ from those of long-term memory consolidation.

The visuo-spatial working memory operations of the dlPFC in monkeys are among the best understood. Much of the data arose from studies using a spatial working memory task termed the oculomotor delayed response (ODR) (Fig. 1). In this task, the monkey fixates on a center spot, while a cue appears briefly in one of eight possible locations. The monkey must remember the cue location over a delay period of several seconds. At the end of the delay period, the monkey makes an eye movement to the remembered location to receive a juice reward. The location of the cue randomly changes from trial to trial, thus requiring constant updating of the contents of working memory. The dlPFC is needed to perform this working memory task, and even small lesions in this area can produce permanent deficits in performance[20]. Neuronal recordings from the dlPFC in monkeys performing a spatial working memory task have found neurons that fire to the Cue and/or to the Response, but also neurons that are able to maintain spatially-tuned, persistent activity across the delay period[21]. This delay-related persistent activity has been considered to be the neuronal mechanism of working memory due to the following features[22]: first, this neuronal activity persists during the time period when a representation needs to be remembered; second, sustained neuronal activity ceases when a memory-guided response has been generated and the representation is no longer needed; third, when activity does not persist throughout the delay period, behavioral performance is compromised; and fourth, the persistent activity is direction-selective. The pioneering work of Goldman-Rakic revealed that this persistent memory-related activity is generated by the recurrent excitation of pyramidal cells interconnecting on dendritic spines in deep layer III of the dlPFC[23]. Computational models have predicted that this persistent memory-related activity requires stimulation of NMDARs rather than AMPARs[24], and that the slow kinetics of GluN2B receptors is particularly well-suited to persistent dlPFC network firing in the absence of sensory stimulation[25]. In

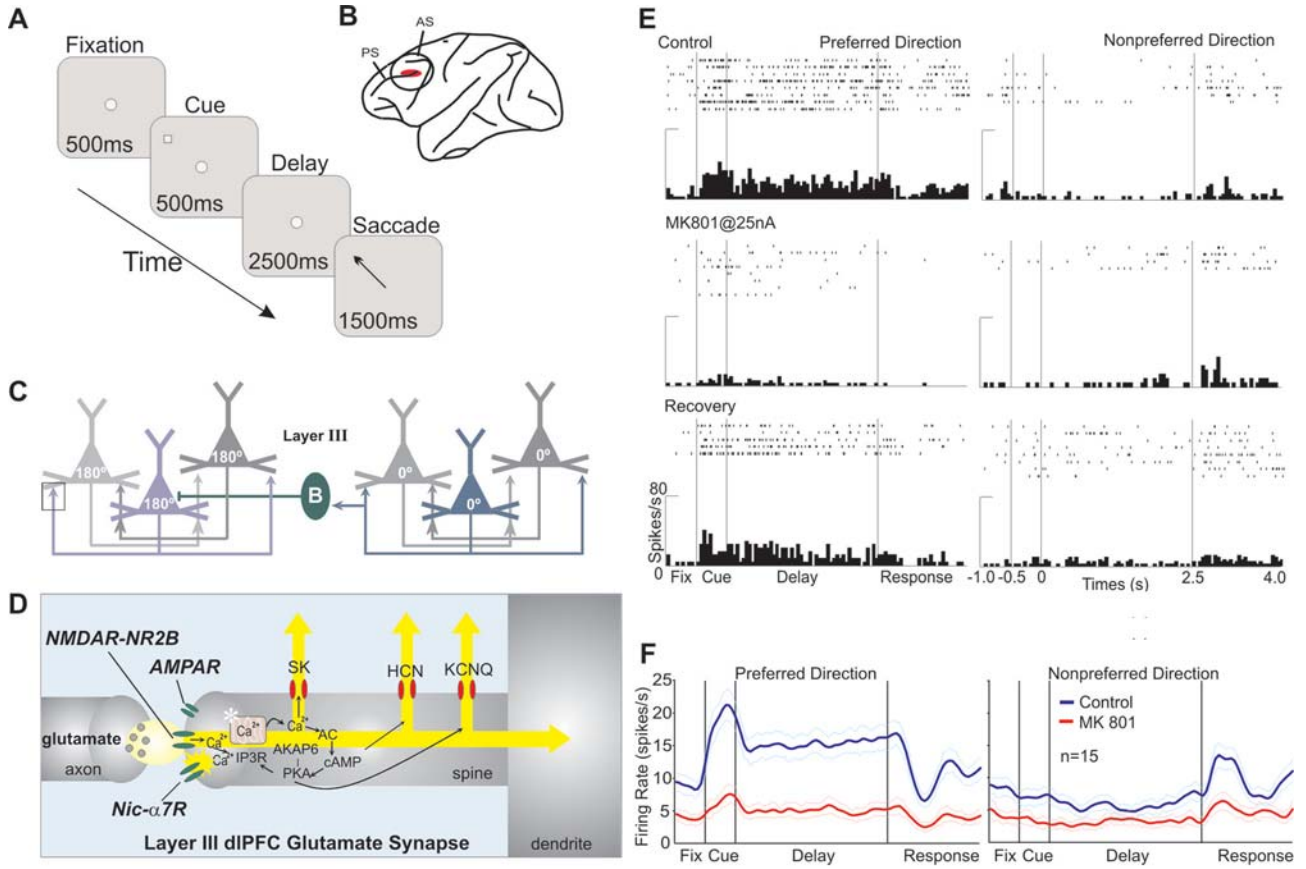

Fig. 1. The actions of NMDARs on the dlPFC neuronal circuitry underlying spatial working memory in primates. A. The spatial oculomotor delayed response (ODR) task. Trials begin when the monkey fixates on a central point for 0.5 s. A cue is presented in 1 of 8 possible locations for 0.5 s, followed by a 2.5-s delay period. When the fixation point is extinguished, the monkey makes a saccade to the location of the remembered cue. The position of the cue changes on each trial in a quasi-random manner, thus requiring the constant updating of working memory stores. B. The region of monkey dlPFC where recordings were made. PS, principal sulcus; AS, arcuate sulcus. C. The deep layer III microcircuits subserving spatially-tuned, persistent firing during the delay period. B, GABAergic basket cell. D. Working model of a glutamate synapse on a spine in layer III of the dlPFC. Glutamate stimulates NMDAR-NR2B receptors in the post-synaptic density, while AMPARs have only subtle actions. Permissive, depolarizing effects for NMDAR actions appear to be mediated by cholinergic stimulation of nicotinic (nic)-α7Rs, which are also localized in the synapse. Ca^{++} entry through NMDAR-NR2B may provide negative feedback by facilitating internal Ca^{++} release from the spine apparatus (asterisk); feedforward Ca^{++}-cAMP signaling opens nearby K^{+} channels to weaken synaptic efficacy and reduce firing. E. An example of an individual dlPFC Delay cell under control conditions and following iontophoresis of the NMDAR antagonist MK801 (25 nA). The rasters and histograms show firing patterns of the neuron's preferred direction and the non-preferred direction opposite to it. Iontophoresis of MK801 markedly reduced task-related firing, which returned towards control levels when delivery of MK801 was stopped (Recovery; *P* <0.05). F. Average responses showing the mean + SEM firing patterns of 15 dlPFC Delay cells for their preferred *versus* non-preferred directions under control conditions (blue) and following iontophoresis of MK801 (red). MK801 markedly suppressed task-related firing, especially for the neurons' preferred direction.

contrast, the faster kinetics of AMPARs leads to dynamic instability and network collapse[24]. Consistent with computational predictions, both *in vitro* and *in vivo* studies have found a prominent role of GluN2B neuronal firing in the PFC. Recordings from rat brain slices have shown more extensive expression of GluN2B in the medial PFC than in the primary visual cortex[26]. A more recent study of the primate dlPFC revealed GluN2B in synapses and that the GluN2B receptor mediates the persistent firing of dlPFC networks in monkeys performing a spatial working

memory task[27]. Immunoelectron microscopy demonstrated that GluN2B is localized exclusively within the postsynaptic densities of layer III dlPFC excitatory synapses on spines, with no evidence of extra-synaptic labeling. Single-unit recordings coupled with iontophoresis in monkeys performing the ODR task showed that the persistent activity of dlPFC neurons is highly dependent on NMDARs, including GluN2B. Iontophoretic blockade of all NMDARs using the antagonist MK801 completely suppresses task-related neuronal firing (Fig. 1). Similarly, blocking GluN2B receptors by iontophoresis of Ro25-6981 produces a marked loss of persistent neuronal firing, and blockade of GluN2A receptors also reduces firing. In contrast, blockade of AMPARs with CNQX/NBQX has only subtle effects on memory-related firing, reducing persistent firing in a small portion of the delay period. AMPAR blockade does alter the firing of sensory neurons in the dlPFC, i.e. it reduces the firing of Cue cells and Post-saccadic Response "feedback" cells. However, the neurons that generate representations of visual space are much more affected by NMDAR than by AMPAR blockade. Interestingly, systemic administration of the NMDAR antagonist ketamine reduces the firing of Delay cells, but increases the firing of Post-saccadic Response neurons (ibid). These results are consistent with the reliance of Delay cells on NMDARs, while the Post-saccadic Response cells have a large AMPAR influence.

If AMPARs have little effect on dlPFC Delay neurons, what depolarizes the membrane and relieves the Mg^{++} block in NMDARs? In the primate dlPFC, these permissive actions appear to be mediated by cholinergic stimulation of nicotinic α7 receptors (nic-α7Rs), rather than AMPARs. Nic-α7Rs are localized in and next to the postsynaptic density in glutamate synapses on spines, and blockade of nic-α7Rs prevents the excitatory actions of NMDA[28]. As acetylcholine is released during wakefulness but not deep sleep, nic-α7R stimulation may permit conscious thought in the waking state. Thus, in the dlPFC, neuronal networks communicate based on arousal state, while in sensory cortex and the hippocampus, NMDAR actions are based on levels of circuit activity, i.e. glutamate release onto AMPARs. Thus, deficits in either NMDAR or nic-α7R signaling weaken dlPFC function.

Finally, Ca^{++} entry through activated NMDARs may contribute to negative feedback to prevent seizures in recurrent excitatory networks. As schematically illustrated in Figure 1, many spines in layer III of the dlPFC contain a spine apparatus, the Ca^{++}-storing endoplasmic reticulum extended into the spine that is elaborated near the synapse. Accumulating evidence indicates that feedforward Ca^{++}-cAMP signaling opens nearby K^{+} channels on dendritic spines to decrease synaptic efficacy and reduce neuronal firing (reviewed in [29]). Future research is needed to determine whether high levels of Ca^{++} entry through GluN2B receptors activate these intracellular pathways.

Relevance to Mental Illness

A variety of cognitive disorders are associated with altered NMDAR signaling. For example, NMDARs are internalized by β-amyloid oligomers in Alzheimer's disease, and this effect occurs in association with nic-α7Rs[30]. Schizophrenia is also linked to genetic insults that weaken NMDAR[31, 32] and nic-α7R[33] signaling. Post-mortem studies have indicated altered GluN2B expression and trafficking[3, 34], including links between allelic changes in GluN2B and impaired reasoning in patients with schizophrenia[35]. There is also accumulating evidence that genetic insults to NMDAR and NMDAR-related synaptic proteins are associated with an increased risk of schizophrenia[32, 36, 37]. The NMDAR antagonist ketamine has been used to model the cognitive deficits of schizophrenia, reducing the blood oxygenation level-dependent response during the delay period of a working memory task in healthy human individuals[38, 39] similar to that seen in patients with schizophrenia[40]. In contrast, the hyperglutamate theories of schizophrenia based on rodent models[41] likely relate to the increased Post-saccadic Response "feedback" cell firing induced by the systemic administration of NMDA antagonists.

In contrast to schizophrenia, where ketamine worsens the symptoms[42], acute ketamine treatment rapidly ameliorates the symptoms in some patients with treatment-resistant depression[43-46], bringing relief within minutes following intra-nasal application[47, 48]. The positive response to ketamine in severely depressed patients has been related to their anterior cingulate response to fearful faces before treatment[49]. Neurons in the anterior cingulate of monkeys have been shown to represent negative emotions

such as symbolic punishment[50], as well as loss of expected rewards[51]. Thus, it is possible that ketamine treatment is helpful in treating depressive symptoms by reducing the firing of NMDAR-dependent, recurrent excitatory circuits in the anterior cingulate and/or in other ventromedial PFC circuits (e.g. Brodmann's area 25[52]) that represent negative emotions and instigate mental suffering. Interrupting the activity of these circuits might underlie the immediate beneficial effects of ketamine in some patients, prior to the regrowth of dendritic spines[53] that may underlie more prolonged beneficial actions. Decreased firing of neurons in the anterior cingulate and area 25 may also underlie the rapid relief of pain by intranasal ketamine (within 5–25 min)[54], as these medial PFC areas are part of the circuits that process the emotional response to painful events[55, 56]. Since intra-nasal ketamine relieves physical pain within minutes[54, 57, 58], it thus may relieve "psychic pain" as well. More research is needed to determine whether NMDARs mediate medial PFC circuits in primates similar to their actions in the dlPFC circuits representing visual space.

Conclusion

New research on the primate dlPFC indicates that GluN2B receptors play a prominent role in the generation of mental representations needed for abstract thought. The data suggest that cholinergic actions at nic-α7Rs are permissive for NMDA synaptic activity, and for the dlPFC network representation of visual space. These data underscore why changes in NMDAR or nic-α7R signaling in diseases such as schizophrenia and Alzheimer's disease have such devastating effects on higher cognition. The unique properties of these dlPFC circuits must be considered in order to design effective treatments for cognitive disorders.

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·Review·

Exploring prefrontal cortex functions in healthy humans by transcranial electrical stimulation

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The prefrontal cortex is involved in a multitude of cognitive, emotional, motivational, and social processes, so exploring its specific functions is crucial for understanding human experience and behavior. Functional imaging approaches have largely contributed to the enhancement of our understanding, but might have limitations in establishing causal relationships between physiology and the related psychological and behavioral processes. Non-invasive electrical stimulation with direct or alternating currents can help to enhance our understanding with regard to specific processes, and might provide future protocols able to improve them in case of malfunctions. We review the current state of the field, and provide an outlook for future developments.

Keywords: affective disorders; brain stimulation; frontal lobe

Introduction

The prefrontal cortex is a compartment of the human brain involved in highly diverse processes, ranging from cognition, motivation, emotion, and complex motor activity to social interactions[1-6]. Disturbances of prefrontal functions are involved in a multitude of neuropsychiatric diseases, including depression, schizophrenia, addiction, dementia, and Parkinson´s disease[7-11]. Thus understanding the complexity of prefrontal physiology is of crucial importance to understand human experience and behavior in health and disease.

Non-invasive imaging approaches, such as functional magnetic resonance tomography, positron emission tomography, and encephalographic (EEG) techniques, have largely facilitated our understanding of human prefrontal functions in the last years. These methods allow identification of cortical activity and excitability changes associated with functions. However, with these techniques it is often difficult to draw conclusions about the causal relationships between the respective processes. To this end, a combination of functional imaging and methods that modulate physiology, such as cortical excitability, activity, plasticity, and oscillations, might be helpful. If modulation of physiological processes results in functional alterations, a causal relationship can be assumed. In the last years, a couple of such stimulation protocols have become available, allowing non-invasive modulation of brain activity and excitability, and thus are principally suited to serve this aim[12-15]. In this review, we give an overview of the principal mechanisms of the tools, and their applications for the exploration of prefrontal functions.

Physiology of Transcranial Electrical Stimulation

Transcranial direct current and alternating-current stimulation (tDCS and tACS) refer to the application of relatively weak currents to the brain *via* scalp electrodes. Specifically, tDCS is the tonic application of constant direct current, and tACS refers to symmetrical oscillatory stimulation. In the case of tDCS, the resulting current flow in the brain induces a subthreshold alteration of neuronal resting membrane potentials, which alters cortical excitability and activity, dependent on the direction of current flow. In the model of the human motor cortex, anodal tDCS enhances, while cathodal tDCS reduces

excitability[16-18]. Whereas the effects of brief stimulation lasting for a few seconds seem to be solely based on membrane potential changes, longer-lasting stimulation for a few minutes induces lasting changes in cortical excitability, which can be stable for about one hour or even longer. These neuroplastic after-effects are assumed to be caused by a change in the strength of glutamatergic synapses, are calcium-dependent[19,20], and thus share some similarities with long-term potentiation and depression, as found in animal studies[21].

The primary mechanism of tACS is assumed to be similar to that of tDCS, namely a sub-threshold alteration of resting membrane potential, whose direction depends on the direction of current flow. Different from tDCS, tACS has no major plasticity-inducing effect[22], although recent studies suggest that exceptions do exist[23]. Modelling and animal and human studies have shown that relatively focal AC stimulation can lead to widespread entrainment of oscillatory activity at the induced frequency[24,25]. The main effect of tACS in humans is a modulation of oscillatory frequency bands in the EEG, if these match the stimulation frequency. For instance, tACS at alpha frequency enhances activity in the visual cortex, and results in excitability alterations[26,27]. Thus the main functional effect of tACS seems to be a modulation of cortical oscillations. In this way, tACS is qualitatively different from tDCS.

Cognitive Functions in the Context of Prefrontal Processing

Working Memory

The dorsolateral prefrontal cortex (DLPFC) is critically involved in working memory, as suggested by task-related activation of this area during performance[28]. In particular, the left DLPFC is relevant for verbal working memory, as explored by testing performance in an *n*-back task with excitability-enhancing anodal tDCS over the left DLPFC. In accordance with the hypothesis, tDCS improved performance, as compared to sham stimulation[29]. In a related working memory task, the beneficial effects of anodal tDCS on performance accuracy developed during stimulation, and were stable for up to 30 min after the completion of stimulation[30]. Zaehle and co-workers[31] described similar positive effects of left prefrontal anodal tDCS on response accuracy in an *n*-back working memory task, while cathodal tDCS disturbed performance. Interestingly, anodal tDCS enhanced alpha and theta activity in parallel, while cathodal tDCS had opposite effects, thus offering a plausible physiological substrate for the effects of tDCS on performance.

While the above studies report accuracy enhancements by prefrontal stimulation, other studies have reported only improvement of reaction time in related tasks[32,33], possibly due to different stimulation protocols (tDCS applied before task performance), or ceiling effects. Recent studies suggest that the specific effects also depend in a non-linear fashion on stimulation intensity[34], inter-individual anatomical and demographic differences[35–37], and task phase (learning *versus* overlearned[38]), and that left prefrontal anodal tDCS can also improve performance in other working memory tasks[39]. Given the performance-related alteration of oscillatory activity[31,40], the contribution of theta activity to working memory performance was explored in subsequent studies. Left dorsolateral prefrontal oscillatory stimulation within the theta frequency range, as well as bilateral stimulation of the DLPFC, improved working memory[40,41]. Moreover, Polania and co-workers have described task-related synchronization in the theta range in the left parietal and prefrontal areas during an *n*-back task. Testing the causal relevance of this synchronization to performance, they showed that synchronized tACS in both areas improved, but desynchronized activation impaired performance (Fig. 1). This effect is specific for the theta frequency band[40]. Therefore it can be concluded that synchronized activity in the theta frequency range between task-related activated areas is critical for working memory performance. A recent study has elucidated more closely the specific contribution of oscillatory activity in the prefrontal cortex to working memory performance, showing that decoding of oscillatory activity in the gamma frequency range allows the identification of stored information[42].

Apart from working memory, the prefrontal cortex also participates in many other cognitive processes such as attention, long-term memory, complex problem-solving, and decision-making. However, the number of studies exploring the contribution of the prefrontal cortex to these functions *via* tDCS/tACS is limited so far.

Attention

Excitability-enhancing tDCS of the left DLPFC has been

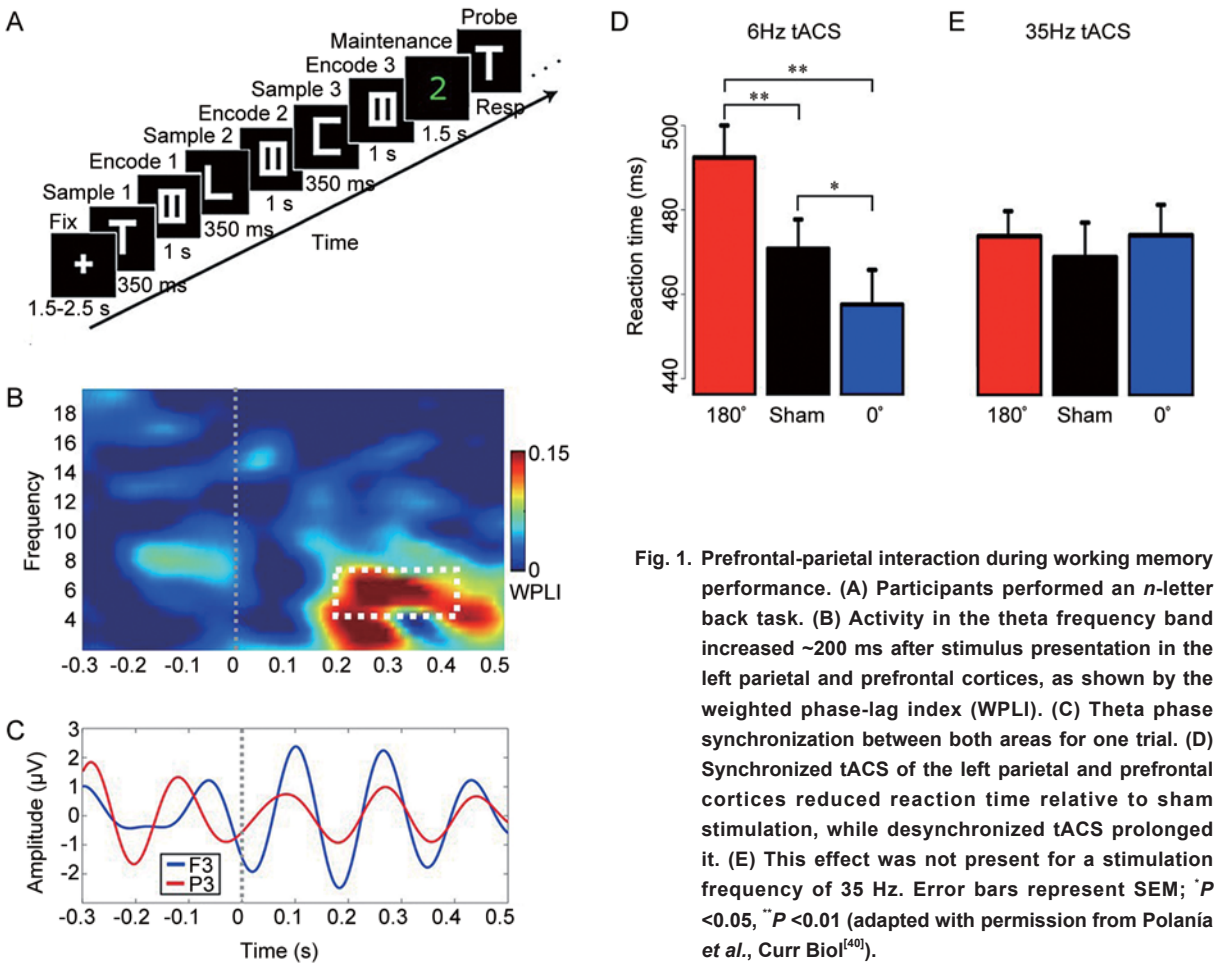

Fig. 1. Prefrontal-parietal interaction during working memory performance. (A) Participants performed an *n*-letter back task. (B) Activity in the theta frequency band increased ~200 ms after stimulus presentation in the left parietal and prefrontal cortices, as shown by the weighted phase-lag index (WPLI). (C) Theta phase synchronization between both areas for one trial. (D) Synchronized tACS of the left parietal and prefrontal cortices reduced reaction time relative to sham stimulation, while desynchronized tACS prolonged it. (E) This effect was not present for a stimulation frequency of 35 Hz. Error bars represent SEM; $^{*}P$ <0.05, $^{**}P$ <0.01 (adapted with permission from Polanía *et al.*, Curr Biol[40]).

shown to improve performance in the Stroop task[39]. Thus this area seems to be involved in attentional set-shifting. In addition, tDCS of the DLPFC seems to have beneficial effects on sustained attention[43]. A recent study showed that anodal tDCS has heterogeneous effects on set-shifting in a parametric Go/No-Go test with regard to the carrier status of the catechol-O-methyltransferase Val158Met polymorphism[44], which provides for the first time evidence for state-dependence of the effect of prefrontal activation on performance.

Long-Term Memory

With regard to long-term memory processes, Javadi and Walsh[45] have described the role of the left DLPFC in word memorization: anodal tDCS improves encoding and trend-wise recognition, whereas cathodal stimulation impairs recognition. In accord, anodal tDCS of this area improves the re-consolidation of learned verbal material[46], and improves performance when applied during word retrieval[47]. These results propose an involvement of the DLPFC in different phases of long-term memory formation and the retrieval of learned material.

Problem-Solving

Some tDCS studies have suggested an involvement of the prefrontal cortex in problem-solving. For example, Cerruti and Schlaug[48] described an improving effect of anodal tDCS of the left DLPFC on complex verbal associative thought. Another study showed that solution recognition of difficult problems is improved by anodal tDCS over the same area[49]. Interestingly, tDCS over the left DLPFC has a performance phase-specific effect in the Tower of London task, which involves strategic planning. In detail, cathodal tDCS improves task performance when applied during the

early acquisition phase, probably due to its reducing effect on distractive cortical noise, whereas anodal stimulation improves performance when applied in the later stages, presumably *via* its activity-enhancing effect on task-related neuronal activity[50]. It has been suggested that prefrontal gamma activity is relevant for performance of this kind of task, and indeed tACS in the gamma frequency range seems to improve fluid intelligence[51].

Decision-Making

Prefrontal areas also seem to be involved in decision-making. Bilateral activity modulation of the DLPFC by tDCS reduces risky behavior in a decision task, most probably by altering bihemispheric activity balance, because unilateral stimulation has no effect[52]. In a related task, however, only anodal right/cathodal left stimulation improved performance[53], which is compatible with a risk-avoiding impact of right prefrontal activity. In older participants however, the same electrode arrangement results in more risky behavior, which is possibly caused by age-dependent differences in prefrontal information-processing[54]. The results of a related study conducted by Pripfl and co-workers[55] show different effects of tDCS in risky decision-making dependent on the inclusion of emotional content and smoking state, which hints at the impact of task characteristics and personality factors on information-processing in the prefrontal cortex. In another risk-taking task, however, the same electrode arrangement did not modulate risky behavior, but enhanced confidence in the decision, which shows that evaluative aspects of a decision are also under prefrontal control[56].

Social Cognition

The prefrontal cortex is also involved in social cognitive processes. Knoch and co-workers[57] have explored the importance of the right DLPFC for performance in the ultimatum game. In this game, a fixed monetary reward has to be split between two participants, one of whom (the proposer) proposes how to split the amount of money, and the other (the responder) can accept or reject the offer. If the responder accepts the offer, he/she gets the money as proposed; if not, he/she gets nothing. The conflicting aspects involved in decision-making are the perception of unfairness and economic self-interest. In line with the hypothesis that the right DLPFC is associated with social decision-making, especially with regard to emotion-based control processes, cathodal stimulation of the right prefrontal cortex, which is involved in the generation of negative emotions, increases the acceptance rate of unfair offers. Recently, the role of the right prefrontal cortex in decision-making was explored in a similar game from the perspective of the proposing participant[58]. The results showed that anodal tDCS of this area improves norm-compliant behavior, but cathodal stimulation selectively reduces it when unfair behavior is expected to be punished by a human counterpart. Interestingly, these behavioral changes are not accompanied by related changes in the rating of fairness, or expected punishment. In addition, these effects are substantially weaker in a non-social scenario version of the game, in which the counterpart is a computer, showing that these effects are specific for social norm-compliant behavior.

Taken together, the results of these studies underscore the role of the prefrontal cortex in a multitude of cognitive functions. So far, the DLPFC has been chosen most often as the target of stimulation, probably because it is relatively easily accessed by non-invasive brain stimulation and has been closely associated with many cognitive processes by functional imaging methods. Exactly how stimulation alters prefrontal information processing has not been explored in much detail so far, maybe with the exception of working memory, and is an important future endeavor. Interestingly, some studies have reported that identical stimulation protocols have distinct effects depending on demographic and personality factors, as well as task characteristics. Given the complex anatomy, physiology, and pharmacology of this brain area, this is not surprising. Closer identification of the contributions of these factors might help to unravel the mechanisms of prefrontal information processing in greater detail in future studies.

Emotional Processes

It is well established that the prefrontal cortex is part of the neuronal networks involved in mood and emotion processing. In healthy individuals, the ventromedial and inferior-medial prefrontal cortex seems to be prominently involved in self-referenced affective state[59,60], whereas the DLPFC is more important for processing stimuli without self-referential emotional content, e.g. faces or visual scenes[61-63]. However, this distinction seems to be gradual

and might reflect the fact that the medial prefrontal cortex is generally more heavily involved in emotional, and the lateral prefrontal cortex in cognitive processing, but both functional properties substantially overlap[60]. In addition, a hemispherical difference in the processing of positive and negative emotional content has been described. Happy mood and positive emotional stimuli induce predominant left DLPFC activity[62,64,65]. Accordingly, lesions of the left prefrontal cortex by stroke, tumors, or epilepsy are often accompanied by depression, while lesions of the right prefrontal cortex are associated with elated mood[66-68]. Also, clinical depression is associated with left DLPFC hypoactivity, while activity of the right prefrontal cortex might be increased[69].

Some tDCS studies have been performed to disentangle the causal contribution of the prefrontal cortex to the experience of emotion, and emotion-related information processing in healthy humans. From their results, tDCS of the DLPFC does not modify mood in healthy individuals[70,71]. With regard to information-processing that includes emotional content, however, the DLPFC seems to be involved. tDCS of the left DLPFC and the right frontopolar cortex improves identification of faces displaying non-neutral mimics independent of stimulation polarity, as compared to sham stimulation (Fig. 2)[70]. Moreover, emotionally aversive faces are rated less unpleasant with anodal stimulation of the left DLPFC[72]. The same stimulation protocol also reduces the emotional valence of negative pictures[73,74]. In the latter studies, this was associated with higher beta and lower alpha EEG activity, and introversion was positively associated with the efficacy of stimulation. For positive affective stimuli, anodal stimulation of the left DLPFC also improves reaction times, and increases the amplitude of relevant event-related potentials[75]. Beyond perceptual and evaluative emotion-associated information processing, the DLPFC seems also to be involved in emotion regulation. In a task in which the participants are instructed to downregulate or upregulate emotional responses to the presentation of negative or neutral pictures, anodal tDCS of the right DLPFC improves the amount of intended emotion regulation[76]. Finally, anodal tDCS over the right DLPFC combined with left frontopolar cathodal tDCS applied in the re-consolidation phase of a fear-conditioning paradigm improves fear memories, which is in accord with an involvement of the prefrontal cortices in fear memory consolidation[77].

In general, the results of these studies support the assumption that prefrontal areas are involved in the processing of emotional information at different levels of complexity, ranging from perception to memory. Further, some pilot studies suggest that relevant alterations are associated with physiological changes in event-related potentials and EEG activity. Most of the studies have been performed with regard to the contribution of the DLPFC. For ventromedial and frontobasal areas that might be more closely associated with emotion generation, no studies are available so far. While this might be due to the fact that these areas are less accessible to non-invasive brain stimulation techniques, this might nevertheless be an important future endeavor.

Concluding Remarks

The prefrontal cortex has been implicated in a multitude of psychological processes, including cognition and emotion. Since functional imaging and EEG approaches are in many cases not well-suited to establishing causal connections between physiological and psychological processes, brain stimulation is a potentially attractive approach to drawing conclusions. tDCS and tACS have been introduced to modulate task-dependent cortical activity and excitability changes. Indeed, many studies in healthy humans have shown that both tools can be used to modulate psychological functions and physiological processes. While the results of these studies have improved our knowledge of prefrontal functions, many questions are still unanswered, and these should be topics for future studies.

Most stimulation protocols so far have explored the functions of the DLPFC, most probably because it is relatively easy to access. The functions of other areas such as the ventromedial or orbitofrontal cortices in emotional processes are also worth studying. Modelling approaches might offer options to tackle these areas more selectively[78]. A related potential shortcoming is the use of relatively large electrodes, and bipolar electrode montages, which limit the specificity of stimulation effects. Also, advanced stimulation protocols, e.g. using smaller stimulation electrodes, large return electrodes, or multiple electrode approaches, might be helpful[79,80]. Moreover, combining measures of task performance with physiological outcome parameters *via*

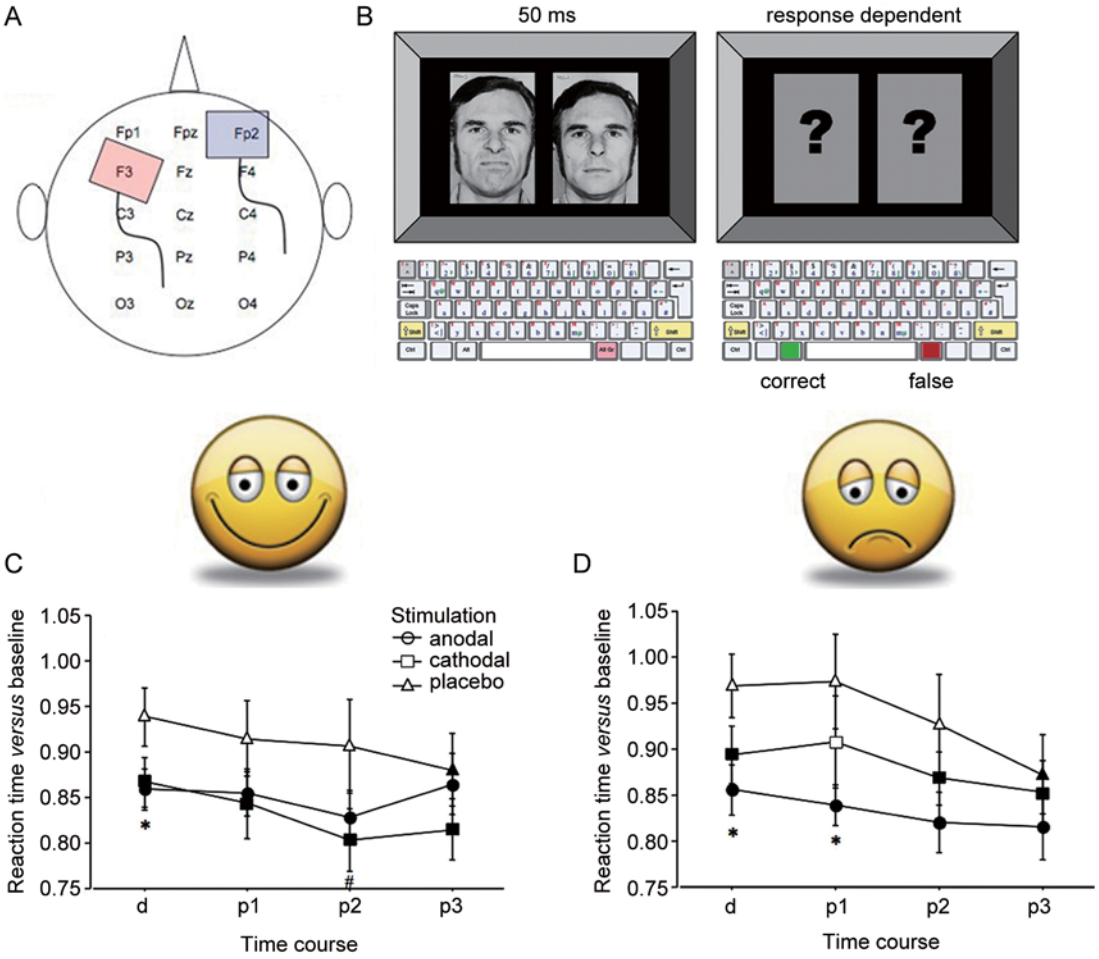

Fig. 2. Alteration of emotion-based information-processing by prefrontal tDCS. (A) tDCS was applied to the left dorsolateral prefrontal and right frontopolar cortex. Polarity refers to the dorsolateral prefrontal electrode. Participants had to identify the position of a non-neutral facial emotional expression as rapidly as possible, and press the appropriate key repetitively before, during, and after anodal, cathodal, or sham tDCS (B). For positive (C), and negative (D) facial expressions, reaction times became faster during the course of the experiment, thus indicating learning of the task in all stimulation and emotional conditions. Under both real stimulation conditions and for both facial expressions, reaction time reductions became significantly faster than with placebo stimulation. For anodal tDCS, positive emotional facial expressions were identified faster than with placebo stimulation during and after tDCS. For emotionally negative facial expressions, anodal tDCS improved perception only during tDCS as compared to placebo stimulation. A minor effect can be seen for cathodal tDCS compared to placebo stimulation (p2 only). Filled symbols: significant reaction time differences relative to baseline values; asterisks: significant differences between anodal tDCS and placebo tDCS; hash symbols: significant differences between cathodal and placebo tDCS for a given time point (paired, two-tailed *t*-tests, $P < 0.05$). Vertical bars indicate standard error of the mean. d, during; p1, immediately and 5 min; p2, 10 and 20 min; p3, 30 and 60 min after tDCS. Adapted with permission from Nitsche *et al.*, Front Psychiatry[70].

simultaneous EEG, ERP, or functional imaging approaches, which is now technically possible, will further enhance our understanding of psychological-physiological interactions. In this connection, functional connectivity approaches might be especially helpful, since the respective psychological functions, and the effects of electrical stimulation, alter network functions[40,81]. An emerging topic might be the elucidation of the foundation of inter-individual differences with regard to the efficacy of transcranial electric stimulation. Here, initial efforts have been made to explore trait- and state-dependency of the effects.

With regard to application aspects, it should be kept

in mind that the studies referred to in this review were not intended to induce maximally strong effects, but aimed to explore the contribution of certain cortical areas to psychological processes. So far, it is unknown to what degree tES can alter psychological functions. Likewise, the impact of tES on performance in a certain laboratory task does not necessarily imply that the same effects are achieved in everyday life, and – maybe more important – whether these effects would be meaningful. This applies also to clinical applications, where pathological changes of cortical excitability, activity, and pharmacology might alter the impact of brain stimulation as compared to healthy humans.

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·Review·

Prefrontal cortex and the dysconnectivity hypothesis of schizophrenia

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Schizophrenia is hypothesized to arise from disrupted brain connectivity. This “dysconnectivity hypothesis” has generated interest in discovering whether there is anatomical and functional dysconnectivity between the prefrontal cortex (PFC) and other brain regions, and how this dysconnectivity is linked to the impaired cognitive functions and aberrant behaviors of schizophrenia. Critical advances in neuroimaging technologies, including diffusion tensor imaging (DTI) and functional magnetic resonance imaging (fMRI), make it possible to explore these issues. DTI affords the possibility to explore anatomical connectivity in the human brain *in vivo* and fMRI can be used to make inferences about functional connections between brain regions. In this review, we present major advances in the understanding of PFC anatomical and functional dysconnectivity and their implications in schizophrenia. We then briefly discuss future prospects that need to be explored in order to move beyond simple mapping of connectivity changes to elucidate the neuronal mechanisms underlying schizophrenia.

Keywords: prefrontal cortex; schizophrenia; anatomical connectivity; functional connectivity

Introduction

Schizophrenia is a debilitating mental disorder affecting ~1% of the general population, with disturbances of cognitive, social, and behavioral functions. A popular hypothesis for this disorder is that schizophrenia is a “dysconnection” disorder and its symptoms are thought not to be due to a single, regionally-specific pathophysiology but to abnormal interactions between regions[1-5]. Recent MRI studies have provided further evidence for this opinion[6-8]. Among the regions implicated in the pathophysiology of schizophrenia, the prefrontal cortex (PFC) has always been of interest[9], due to changes in neurodevelopment processes, abnormalities in anatomy and function, and its role in the cognitive functions that are impaired in schizophrenia[10]. Recent network analyses based on graph theory have also revealed that the PFC is one of the hub regions affected in schizophrenia[11]. However, no area of the brain acts in isolation. To understand the implications of the involvement of the PFC in schizophrenia, we need to understand the PFC in the context of the brain as a whole. In this review, we summarize the major advances in the anatomical and functional connectivity of the PFC in schizophrenia to generate a clear picture of how PFC dysconnection relates

to this disorder. Then, we discuss current challenges and future research directions.

A Brief Introduction to the PFC

The PFC plays an essential role in the organization and control of goal-directed thought and behavior[12]. Specifically, the lateral PFC is critical for the selection, monitoring, and manipulation of cognitive task sets; the medial PFC is critical for updating these sets; and the orbitofrontal cortex (OFC) is critical for assigning social and emotional meaning to these sets in order to better guide goal-directed behavior[12] (see reference[12] for a detailed introduction to the specific function of each PFC area). Furthermore, the extensive reciprocal connections between the PFC and nearly all cortical and subcortical structures, especially the limbic regions, place it in a unique position to orchestrate a wide range of cognitive and affective neuronal functions[12]. The architectonic subdivisions of the PFC and the major PFC white-matter tracts involved in schizophrenia are illustrated in Figure 1.

Based on the unique role of the PFC in normal functioning, research has linked it with schizophrenia. The major findings in schizophrenia include: spine loss and dendritic atrophy of PFC neurons; smaller PFC grey matter volume; profound dysfunction of the PFC (including deficits in working memory); and changes in gene expression (for review, see[15]). Among these, the changes in microcircuits of the PFC in schizophrenia suggest the possibility of altered connectivity between the PFC and other regions[15].

Anatomical Dysconnectivity of the PFC in Schizophrenia

Evidence from myelin pathology in postmortem brain tissue and gene expression profiling has shown that anatomical connectivity might be pathologically changed in schizophrenia[16]. Diffusion tensor imaging (DTI), a new and powerful tool, affords the possibility to explore the anatomical connectivity in the human brain *in vivo*. By measuring the degree of anisotropy in the random motion of water molecules, DTI can quantify and visualize white-matter fiber tracts[17]. Fractional anisotropy (FA) is the most-commonly used DTI index[18] to examine white matter

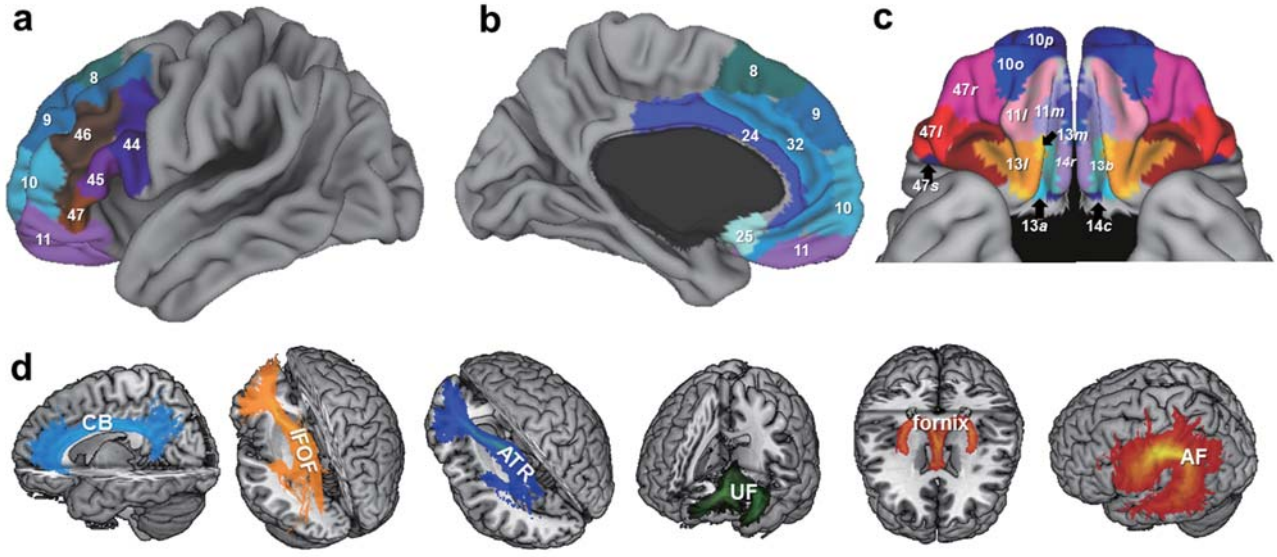

Fig. 1. Architectonic subdivisions of the PFC (a, b, c) and the major PFC white matter tracts involved in schizophrenia (d)[13, 14]. Dorsolateral PFC: lateral area 8, lateral area 9, and area 46; ventrolateral PFC: areas 44, 45, and 47; rostral PFC: area 10; orbitofrontal cortex: areas 11 (11m and 11l), 13 (13a, 13b, 13m, and 13l), 14 (14r and 14c), 10 (10p, 10o), and 47/12 (47/12r, 47/12m, 47/12l, and 47/12s); medial PFC: medial areas 8, 9, 10, 32, 24, and 25. The major white-matter tracts linking the PFC and other brain regions are the cingulum bundle (CB), inferior fronto-occipital fasciculus (IFOF), anterior thalamic radiation (ATR), uncinate fasciculus (UF), fornix, and arcuate fasciculus (AF), all of which are implicated in schizophrenia.

integrity. Since the seminal work[19] in which DTI was first applied to schizophrenia, studies have repeatedly found white-matter pathology in schizophrenia by region of interest (ROI) measures to define the fiber tracts, by voxel-based analysis, and by fiber tractography[20]. A systematic meta-analysis of voxel-based DTI FA studies of patients with schizophrenia revealed significant reductions in the left frontal and left temporal deep white matter[21]. The region in the left frontal deep white matter is traversed by tracts interconnecting the frontal lobe, thalamus, and cingulate gyrus. The region in the temporal lobe is traversed by tracts interconnecting the frontal lobe, insula, hippocampus, amygdala, and temporal and occipital lobes[21]. Similar findings were obtained when analyzing studies on patients with first-episode schizophrenia, in whom reduced FA in the white matter of the right deep frontal and left deep temporal lobes was found[22]. Fiber tracking showed that the main tracts involved are the cingulum bundle (CB), the left inferior longitudinal fasciculus, the left inferior fronto-occipital fasciculus, and the interhemispheric fibers running through the corpus callosum[22]. All of these findings provide evidence for disrupted anatomical connections in the fronto-limbic circuitry, even at the early stages of schizophrenia. Therefore, we focus on several major white-matter tracts linking the PFC and limbic regions, the CB, uncinate fasciculus (UF), and arcuate fasciculus (AF) to understand the clinical correlates of PFC anatomical dysconnectivity in schizophrenia.

Cingulum Bundle

The CB connects paralimbic-neocortical regions and also interconnects limbic structures including the dorsolateral prefrontal cortex (DLPFC), cingulate gyrus, parahippocampal gyrus, and amygdala[20]. The CB is involved in a number of functions, including emotion, self-monitoring, and spatial orientation and memory. By placing ROIs on the CB, Kubicki and coworkers reported reduced FA in the CB in schizophrenic patients compared with controls[23]. This finding has been repeatedly replicated by different methods including ROI analysis, voxel-based analysis, and fiber tractography[23-29]. Decreased FA in the CB has been linked with various cognitive dysfunctions in schizophrenia, such as errors in executive functions relevant to performance monitoring[23], poor general intelligence and working memory[30], impaired Stroop performance[31], and increased saccadic latency[32]. In addition, higher FA in the left CB and left fronto-occipital fasciculus is associated with lower within-individual variability for speed on a computerized neurocognitive battery in healthy controls, but not in patients with schizophrenia[33].

Uncinate Fasciculus

The UF is a bidirectional, long-range white-matter tract that connects the lateral OFC and Brodmann area 10 with the anterior temporal lobes[34]. One would expect that the UF connecting limbic regions to OFCs might be structurally impaired in schizophrenia. However, a recent review of the DTI literature indicates that findings on FA in the UF in schizophrenia are mixed. The UF appears to play either a small role, or no role, in this disorder[34]. Clinical heterogeneity combined with small sample sizes may account for the contradictory results. It is possible that the integrity of the UF is correlated with specific symptoms of schizophrenia. Two studies have shown that the FA values in the left UF of schizophrenic patients with deficit syndromes (such as flattened affect and lack of social engagement) are lower than those of non-deficient patients and controls[35, 36]. In addition, the severity of deficit symptoms is strongly correlated with disruption of the same tract in a group of patients with first-episode schizophrenia[36].

Arcuate Fasciculus

The AF bidirectionally connects the caudal temporal and inferior parietal cortices to the frontal lobe. Due to the fact that this tract connects Wernicke's and Broca's areas, the AF is the major language-processing tract in the brain. Therefore, the integrity of the AF is often linked with the language and thought disturbances in schizophrenia[37]. Although mixed findings have been reported[37, 38], more consistently a reduced FA value in the AF has been found in schizophrenic patients with auditory verbal hallucinations[39-41]. Several studies have also suggested that changes in the integrity of the AF may be relevant to the risk of developing psychosis; decreased axonal or fiber integrity has been reported in the AF of siblings of patients diagnosed with schizophrenia[42, 43]. The disrupted integrity in the AF is consistent with the evidence of language deficits in those at familial or a clinically increased risk for schizophrenia[42].

Several other white-matter tracts connecting the PFC and other regions have also been investigated. These tracts include the fornix, a tract connecting the hippocampus with other regions including the PFC. This tract is important in spatial learning and memory, which are disrupted in schizophrenia. Disrupted integrity of the fornix has been found in a group of patients with schizophrenia, who also showed disrupted functional connectivity between the hippocampus and other regions implicated in episodic memory, such as the medial prefrontal cortex (MPFC)[44]. Another tract that shows FA reduction in schizophrenia is the inferior fronto-occipital fasciculus (IFOF), which connects the occipital, posterior temporal, and orbitofrontal areas[45]. Decreased FA in the left IFOF predicts worse neurocognitive performance both in never-medicated chronic schizophrenia[46] and in adolescents with early-onset schizophrenia[47], as well as predicting empathic impairments in patients with schizophrenia[48]. Other white-matter fiber tracts related to the PFC in schizophrenia include the anterior limb of the internal capsule, the medial portion of which includes the anterior thalamic radiation linking the thalamus and the PFC[49, 50], the genu of the corpus callosum linking the bilateral PFCs[51, 52], and deep white matter within the PFC[53].

In addition, globally exploring changes across the entire brain using graph-based network analyses provides a means of searching for possible lesions or alterations in the anatomical connectivity network[17]. By examining networks derived from diffusion imaging data, a longer average path-length and corresponding reduction in global communication efficiency have been found in patients with schizophrenia[11]. Node-level investigation has further revealed altered connectivity centered on frontal association regions[8]. And regional efficiencies in the frontal association cortex are negatively correlated with the severity of symptoms as measured by the Positive and Negative Syndrome Scale[8].

Functional Dysconnectivity Related to the PFC in Schizophrenia

Resting-state functional magnetic resonance imaging (fMRI) and task-based fMRI are often used to make inferences about the connections between brain regions[54]. Using different functional connectivity (FC) analyses, researchers can investigate the PFC-related networks based on single ROIs, specific networks, and whole-brain networks. Abnormal functional interactions between the PFC and widely-distributed regions, such as the parietal cortex, temporal regions, and regions in the default mode network, have been found in schizophrenia both during rest and during several cognitive tasks such as working memory tasks, continuous performance tasks, and reaction-choice tasks (for review, see [55]). Here, we focus on selected networks to illustrate how functional dysconnectivity of the PFC is linked with the impaired cognitive functions and/or the psychotic symptoms of schizophrenia.

Frontostriatal Circuit

Functional dysconnectivity between the PFC and dopamine-regulating regions in the basal ganglia (BG) has been hypothesized to account for two core features of schizophrenia, cognitive deficits and psychosis, based on the dopamine hypothesis of schizophrenia[56], which has been tested in several recent studies[57-59]. Using resting-state fMRI, Salvador *et al.* found increased connectivity between the DLPFC and the BG across low-, medium-, and high-frequency bands, indicating that DLPFC-BG functional dysconnectivity is an abnormal part of the frontostriatal loop in schizophrenia[57]. Yoon *et al.* found task-evoked hyperactivity in the substantia nigra that occurred in association with hypoactivity of the right inferior frontal gyrus (IFG) and the bilateral caudate during a working memory task in a schizophrenic group[58]. They further found decreased FC between the PFC (localized to the right inferior/middle frontal gyrus) and BG regions (substantia nigra and caudate) in patients with schizophrenia while they were performing a working memory task. Similarly, decreased performance-related FCs between the ventrolateral PFC and the bilateral putamen were found during a working memory task, suggesting that weaker frontostriatal connectivity underpins the impaired information retrieval in schizophrenia during working memory performance[59]. Although these studies revealed an abnormality in the frontostriatal circuit in schizophrenia, it is worthy of note that the pattern of abnormality is incompatible: increased FC in the frontostriatal circuit during rest but decreased FC during the task. In order to understand the link between the two types of functional

dysconnectivity, studies measuring resting-state FC and task-state FC in the same individual need to be performed.

Frontotemporal Functional Connectivity

Frontotemporal dysconnectivity has been proposed as a mechanism leading to the psychotic symptoms, especially auditory hallucinations, in schizophrenia. Since the first study suggesting that reduced FC between the left DLPFC and the left superior temporal gyrus was linked to auditory hallucinations in schizophrenia[60], several studies have verified the relationship between frontotemporal functional disconnectivity and auditory hallucinations during different tasks, suggesting a source-monitoring impairment (for review, see[61]). Resting-state FC studies suggest that elevated frontotemporal FC makes auditory hallucinations worse, especially indicated by positive correlations between the reality of hallucinations and the strength of the FC between the left IFG (including Broca's region) and the auditory cortex, posterior temporal lobe, ventral striatum, and anterior cingulate cortex[62]. Based on their recent studies, Hoffman and colleagues proposed a complex functional loop, which includes Wernicke's area and its right homologue, the left IFG, and the putamen, to interpret the generation of auditory verbal hallucinations. In this model, intact FC between Wernicke's area and the left IFG and FC between the left IFG and putamen appeared to allow hyperconnectivity between the putamen and Wernicke's area to be expressed as conscious hallucinations of speech[61]. However, whether the resting-state frontotemporal FC is decreased or increased in schizophrenic patients with auditory hallucinations compared to patients without such hallucinations or healthy participants remains to be determined.

Frontoparietal Functional Connectivity

Functional interactions between the dorsal frontal and parietal regions are engaged by a wide range of higher-level cognitive tasks and are thought to be involved in adaptive task control[63, 64]. In general, greater FC between the dorsal frontal and parietal regions predicts better performance. Disrupted dorsal fronto-parietal FC may account for the impaired executive function and cognitive control in schizophrenia, especially the well-known working memory deficit (for review, see[55, 65]). In addition, the dysfunctional connectivity of the dorsal frontal-parietal network has been correlated with psychotic symptoms such as disorganization[66, 67], which may be due to the disrupted executive function and cognitive control in schizophrenia.

PFC-Hippocampus Functional Connectivity

Disturbed interactions between the PFC and hippocampus have also been proposed to account for the cognitive deficits related to working memory in schizophrenia[68]. Meyer-Lindenberg and colleagues reported persistent undiminished FC between the right DLPFC and left hippocampus in the context of a working memory task in schizophrenia[69]. Benetti and colleagues found that the normal pattern of effective connectivity from the right posterior hippocampus to the right IFG is significantly decreased in both first-episode patients and individuals at high risk for psychosis during a delayed matching-to-sample task, suggesting that a disruption of bottom-up hippocampal–prefrontal integration may be correlated with increased vulnerability to psychosis rather than an effect of chronic illness or its treatment[70].

Medial PFC and the Default Mode Network

The functional connectivity of the MPFC is also involved in psychosis and the cognitive deficits in schizophrenia, due to its role in self-referential mental activity and the organization of thoughts and actions according to internal goals[71]. Hyperconnectivity between the MPFC, a region with reduced task-related suppression during a working memory task, and other regions of the default mode network during both rest and working memory tasks is correlated with the more serious positive symptoms in schizophrenic patients[72]. Moreover, the hyperactivity in the MPFC (reduced task-related suppression) and the hyperconnectivity between the MPFC and the regions of the default mode network during a working memory task are correlated with inferior working memory performance both in schizophrenic patients and their unaffected relatives[72]. These findings suggest that the abnormal MPFC FC may contribute to the disturbances of thought in schizophrenia, impaired working memory performance, and to the risk for the illness[72]. Besides its implications for working memory, the altered MPFC FC is also involved in other cognitive functions. For example, dysconnectivity between the MPFC and the left superior temporal gyrus during a self-other source monitoring task is implicated in the impaired reality monitoring in schizophrenia[73]. And decreased negative connectivity between the MPFC and medial-temporal

regions during perspective-taking has been reported in patients with schizophrenia, and this deficit fully mediates the behavioral impairments in theory of mind in patients[74]. However, similar to other PFC regions, inconsistent findings also exist in the MPFC FC patterns. For example, contrary to hyperconnectivity, a study using an ROI in the ventral MPFC showed decreased resting-state FC between the ventral MPFC and the default mode network regions (such as the anterior MPFC, right middle temporal lobe, hippocampus, parahippocampus, and amygdala) in chronic schizophrenic patients. And the decreased FC between the ventral MPFC and right medial-temporal regions has been correlated with the poorer regulation of emotion[75]. The inconsistency may result from the differences in FC analysis methodology (such as the selection of ROI) and the heterogeneity of schizophrenia.

In general, decreased FCs related to the PFC in schizophrenia are often found when a task is performed; however, both decreased and increased PFC functional dysconnectivities are found in schizophrenia during rest. Most of these abnormal FCs are related to the dorsal PFC, although different circuits are involved. These abnormal PFC connectivities have been implicated in the pathophysiology and pathopsychology of schizophrenia, especially the psychotic symptoms and impaired cognitive functions (such as impaired working memory) (Table 1).

Perspectives

Despite the current knowledge on the clinical correlates of PFC dysconnectivity in schizophrenia, many challenges still exist. Here, we list some challenges and express our opinions about how to address these challenges in order to move beyond simple mapping of connectivity changes to elucidate the underlying neuronal mechanisms of the pathogenesis and pathophysiology of schizophrenia.

Fine-Grained Parcellation of the PFC

The PFC has a heterogeneous cytoarchitecture and functions. It is composed of several cytoarchitectonically different subregions involved in a variety of functions and this suggests the existence of functional subregions, such as the superior frontal gyrus[50]. Even though Brodmann-defined brain areas have their unique internal structure, such as the frontal pole (i.e. Brodmann area 10), functional subregions are also suggested due to the distinct anatomical and functional connectivity patterns[46]. Based on the cytoarchitecture, the distribution patterns of multi-receptor, co-activation patterns, and anatomical and/or functional connectivity, each of the PFC regions (DLPFC, ventrolateral PFC, MPFC, OFC, and frontal pole) has been parcellated into subregions[76-82]. These finer parcellation patterns have important implications for identifying the specific functional role of each subdivision in the PFC.

Despite the complexity of functional regions/subregions of the PFC, the existing studies often selected a roughly-defined PFC region to investigate the differences in connection patterns between schizophrenic patients and healthy controls, or interpreted their findings in a way lacking detailed information on the PFC subregions. There is no doubt that new knowledge on PFC dysconnectivity in schizophrenia will be warranted by the next generation of brain atlases, such as the Brainnetome atlas[83], which has

Table 1. Major findings of PFC functional dysconnectivity in schizophrenia

Region-region	Resting-state FC	Task-state FC	Clinical implications
PFC-BG	↑	↓	Impaired working memory
IFG-temporal lobe	unclear	↓	Reality of auditory hallucination
DLPFC-parietal lobe	↑↓	↓	Deficits in executive function and cognitive control (e.g., working memory); psychotic symptoms
DLPFC-hippocampus	unclear	↓	Impaired working memory; psychosis
MPFC-DMN	↑↓	↑↓	Impaired working memory, reality-monitoring, theory of mind; psychosis

BG, basal ganglia; DLPFC, dorsolateral prefrontal cortex; DMN, default-mode network; FC, functional connectivity; IFG, inferior frontal gyrus; MPFC, medial prefrontal cortex; ↑increase; ↓decrease.

finer parcellation of the PFC and other brain regions[84-87]. On one hand, using a more fine-grained PFC parcellation scheme as a reference for reporting localization results in future studies will be helpful in reducing the confusion in the nomenclature (e.g., lateral PFC, dorsolateral PFC) and make it easier to compare results from different studies, and so will be useful for advancing our understanding of PFC pathophysiology in schizophrenia. On the other hand, it will be possible to identify the neuronal correlates of a specific symptom or an impaired cognitive function with a specific PFC subregion and thus may generate a symptom classification atlas, providing insights into the etiology and pathogenesis of schizophrenia.

However, it needs to be noted that inconsistency exists in the parcellation results obtained by different criteria, methodologies, and imaging modalities. For example, area 44 of Broca's region in the left inferior frontal gyrus can be parcellated into 5 subregions based on its co-activation pattern across different fMRI studies[77], but into 2 subregions based on the distribution pattern of multiple receptors[76], resting-state fMRI-based parcellation[88], and diffusion-weighted tractography-based parcellation[80]. Even using the same methodology, inconsistent findings can be obtained, such as the frontal pole parcellation based on anatomical connectivity obtained by diffusion-weighted tractography[78, 82]. One possible reason for such inconsistency is that there is no standardized protocol to manually identify ROIs of the PFC as targets of the parcellation scheme[55]. Studies are urgently needed to examine the relationships among different parcellation criteria and distinct imaging modalities, and finally achieve a reliable and reproducible map of the human PFC.

Anatomical Basis of Abnormalities in PFC Functional Connectivity in Schizophrenia

The anatomical substrate of functional connectivity has been an active topic of research. By measuring resting-state FC using fMRI and anatomical connectivity using DTI tractography in the same individuals, spatial consistency between anatomical and functional connectivity has been reported in some networks, such as the default-mode network (e.g., between the MPFC and the posterior cingulate cortex), the salience-processing network, and bilateral parietal–frontal task-activation networks in healthy populations[89-91]. Critically, connections among a spatially distributed and topologically central collective called the “rich club” are central to the integration of information among the different functional networks of the human brain[89]. These studies showed that FC is constrained by anatomical connectivity; however, they are not isomorphic. In general, FC is more prevalent than anatomical connectivity. And FC is context-dependent and easily changed, but anatomical connectivity is relatively stable[92]. Researchers have also begun to seek to understand the anatomical basis of aberrant FC in schizophrenia by combining DTI with fMRI. Both decreased and increased FC have been found in patients who show impaired integrity of white-matter tracts or altered structural network topology (for review, please see [92-94]). Decreased structural interconnectivity among rich club hubs (including the bilateral precuneus, superior frontal cortex, superior parietal cortex, and insula) may underlie the broad range of functional network abnormalities in patients with schizophrenia[95], and this may result in the altered functional dynamics and impaired global brain functioning. Although these findings are important, some open questions remain, such as whether anatomical dysconnectivity and functional dysconnectivity in schizophrenia share common biological substrates (e.g., common genetic factors); whether anatomical dysconnectivity and concomitant changes in FC in schizophrenia develop with progression of the illness; how increased FC between regions along with deficits in anatomical connectivity in schizophrenia can be understood; and whether increased functional connectivity has implications for the pathophysiology of schizophrenia or merely results from artifacts in different analyses applied to DTI and fMRI. All of these questions need to be explored. In addition, technical challenges, such as how to resolve crossing fibers and how to better detect relatively small fiber bundles[94], need to be solved.

Genetic Basis of the PFC Dysconnectivities in Schizophrenia

Impaired PFC function and structure have been found more frequently in unaffected relatives of schizophrenic patients, such as unaffected monozygotic twins, than in control individuals without such a family history[96]. This suggests that dysfunction of the PFC in schizophrenia may be controlled by genetic factors. However, it is unclear that PFC dysconnectivity in schizophrenia is also influenced

by genetic factors, due to the lack of twin and family studies that focus on functional or anatomical connectivity. Nevertheless, in healthy populations, pedigree studies have found evidence that genes control the functional and anatomical connectivity of the PFC[97, 98]. For example, Karlsgodt *et al*. found that the integrity of several white-matter tracts related to the PFC (anterior limb of the internal capsule, CB, superior fronto-occipital fasciculus, and superior longitudinal fasciculus) is heritable; furthermore, the integrity of the superior longitudinal fasciculus (a primary frontoparietal connection) shares genetic factors with performance of working memory, a heritable trait relevant to schizophrenia[97]. This evidence suggests that PFC functional and anatomical connectivity may be candidate endophenotypes of schizophrenia[99]. Therefore, some studies have sought to link PFC functional and anatomical connectivity with susceptibility genes for schizophrenia using a strategy called as imaging genetics. For instance, Liu *et al*. reported that the catechol O-methyltransferase (*COMT*) val158met polymorphism significantly modulates prefrontal-related FC within the default mode network because COMT plays a unique role in regulating prefrontal dopamine levels[100]. Liu *et al*. found that the functional and anatomical connectivity of the thalamus to the prefrontal cortex is impacted by *DISC1* (Disrupted-In-Schizophrenia 1) Ser704Cys[101]. Wang *et al*. found that carriers of the *KIBRA* (kidney and brain expressed protein) C-allele have a smaller gray-matter volume in the MPFC and bilateral dorsal anterior cingulate cortices and show higher functional synchronization in the same regions than T-allele homozygotes[16]. Given the complexity of the molecular genetics of cognitive function subserved by the PFC and the complexity of the genetic etiology of schizophrenia, understanding the mechanisms by which genetic variations that are associated with risk for schizophrenia impact PFC functional and anatomical connectivity remains a clinically important challenge. Some efforts have been made by exploring how interactions of multiple genes from the same signaling pathway (e.g., *COMT* and *DRD2* interaction[102]) affect resting-state FC. However, more data from imaging genetics in patients are needed. In addition, studies with non-invasive neuroimaging technologies, such as fMRI and dMRI, cannot clearly elucidate whether PFC dysconnectivity is related to the etiology of schizophrenia and how genes, the brain, and the disorder interplay. Genetically-modified animals are considered good models for the solution of these issues and some efforts are in progress[103]. By using genetically-modified mouse models of schizophrenia, researchers will be able to go beyond neuroimaging to look into the underlying mechanisms with disease-specific behavioral tests as well as gene-specific histological examinations, using interactive investigations that are not possible in human studies. Neuroimaging studies on genetically-modified mouse models of schizophrenia are likely to realize a relatively seamless translation of findings to this disorder, since neuroimaging allows the same biological target to be investigated in both humans and animals. Several novel genetic modification technologies have been developed recently. The CRISPR-Cas9 method is a breakthrough that can rapidly and efficiently generate transgenic mice with multiple modified alleles by direct injection of both single-guide RNA and mRNA encoding Cas9 into embryos[104, 105]. Using this novel technology, known schizophrenia-associated mutations can be introduced into mice and their effects on the brain investigated. Several genome-conserved, specified, and verified genetic mutations associated with the PFC and schizophrenia, such as *COMT*(Val158Met) and *DISC1*(Ser704Cys), may be candidates for this novel technology.

PFC Connectivity-Guided Non-invasive Brain Stimulation for Schizophrenia

Non-invasive brain stimulation techniques, such as transcranial magnetic stimulation and transcranial direct-current stimulation, have been shown to play a role in the non-invasive treatment of schizophrenia, especially for auditory hallucinations[106]. However, researchers have always been concerned about the precision of these techniques and their influence on other brain regions or networks[107]. A promising direction is to combine them with neuroimaging techniques in the context of the Brainnetome atlas. This atlas, with details of the subregions in the PFC and their connectivity patterns, will provide an accurate guide for the location of brain stimulation techniques and *a priori* knowledge of the possible effects of simulating a specific brain region. The combination of brain stimulation and neuroimaging techniques makes it possible to identify the causal effect of brain stimulation on brain activity of

interest in the stimulated region, which has important consequences for the interpretation of the effects of such stimulation. And this combination also provides a particular window into the effects of focal brain stimulation on remote, functionally connected brain regions[108]. In addition, armed with knowledge of the putative causal interactions among brain regions obtained by effective connectivity analyses (e.g., dynamic causal modelling[109]), it is possible to test the behavioral relevance of an effective fMRI connectivity network underlying a cognitive process by using brain stimulation to stimulate the regions identified by effective connectivity[107]. These advances will shed light on the causality behind the PFC dysconnection and symptoms/impaired cognitive functions in schizophrenia, and provide objective valuation for the non-invasive treatment of schizophrenia.

In summary, the variability in the PFC dysconnectivity patterns across patients is associated with the severity of both cognitive impairments (such as impaired working memory) and cardinal symptoms (such as auditory verbal hallucinations), suggesting that these distinct patterns of connectivity might differentially contribute to schizophrenic symptoms. However, the current roughly-defined PFC subregions hamper precise location of these impairments and symptoms. Future studies with fine-grained parcellation of the PFC may provide a clearer understanding of the PFC in schizophrenia. Furthermore, the anatomical and genetic bases of PFC dysconnectivity in schizophrenia need to be determined. The causality behind the PFC dysconnection and symptoms/impaired cognitive functions in schizophrenia needs to be clarified. Further understanding of the implications of PFC dysconnectivity for schizophrenia may benefit from the integrated knowledge in the Brainnetome atlas, multimodal imaging techniques, imaging genetics, and genetically-modified animal models in the framework of the Brainnetome[83].

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·Review·

What can atypical language hemispheric specialization tell us about cognitive functions?

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Recent studies have made substantial progress in understanding the interactions between cognitive functions, from language to cognitive control, attention, and memory. However, dissociating these functions has been hampered by the close proximity of regions involved, as in the case in the prefrontal and parietal cortex. In this article, we review a series of studies that investigated the relationship between language and other cognitive functions in an alternative way — by examining their functional (co-)lateralization. We argue that research on the hemispheric lateralization of language and its link with handedness can offer an appropriate starting-point to shed light on the relationships between different functions. Besides functional interactions, anatomical asymmetries in non-human primates and those underlying language in humans can provide unique information about cortical organization. Finally, some open questions and criteria are raised for an ideal theoretical model of the cortex based on hemispheric specialization.

Keywords: functional lateralization; hemispheric specialization; language production; cognitive functions; co-lateralization

Introduction

Neuroimaging studies in the last years have defined many functionally-specialized brain regions. However, specialization alone cannot fully account for most aspects of brain function. Cognitive functions require the integration of distributed neuronal activity. One task may activate many cortical regions, and one region may be involved in many processes. For example, several important functions, such as attention, working memory, cognitive control, and language production, are critically dependent on the prefrontal cortex. Yet, anatomical architecture[1] and functional experience seem to create regularities in cortical organization across subjects. Functional ontologies can chart the complex relationship between anatomy and function by depicting which sub-process causes the activation of which precise anatomical region and *vice versa*[2], provided that both anatomical networks and task contexts are dynamic. Recently, a many-to-many approach was presented because functions not only seem to be rooted in distributed networks, but configured circuits also interact with each other[3]. Some recent studies have modeled the brain as graphs consisting of different functional networks, and these studies converged on a set of fundamental attributes of human brain organization, in line with those found in nonhuman primates[4,5]. We argue that the way the cortex is organized (be it according to a one-to-one, one-to-many, or many-to-many mapping between anatomy and function) can be uniquely investigated by looking at the anatomical and functional correlates of (a)typical lateralization of language. It is well-documented how behavioral tasks and handedness can help identify subjects with (a)typical speech dominance, and there is a rich neuroimaging literature on the sub-

processes of language. Moreover, language seems to be the most pronounced lateralized function so far. In this review, we further discuss studies that have used (a)typical speech dominance to explore the organization of other language-related and non-language functions. Subjects with atypical lateralization allow investigation of what consequences a shift in one function has for other networks that do not seem to be related at first sight. As such, anatomical and functional relationships that are otherwise difficult to dissociate can be mapped in a healthy population.

Functional Lateralization of Language and Handedness

The capacity for language is unique to human beings. Its well-documented lateralization makes it an even more intriguing function. Left hemisphere (LH) dominance for language production is a robust finding at the population level. In the 19th century, Marc Dax and Paul Broca first reported that speech problems are more likely to occur after injuries to the frontal part of the left hemisphere than after injuries to the right hemisphere (RH). Some early evidence for language dominance came from split-brain patients, whose corpus callosum was sectioned to control intractable epilepsy. The seizures were decreased by disconnecting the two hemispheres. Testing of each disconnected hemisphere in split-brain patients seems to show quite extensive language understanding in the isolated RH, but no speech output[6]. These results therefore suggest the dominant role of the LH in language production, and this hemispheric specialization has been supported by a wealth of evidence from neuroimaging studies in the last two decades, for the great majority of individuals.

Similar to the population-level bias towards LH dominance for language, a strong bias towards the right hand at the population level has probably existed for more than ten thousand years[7]. A popular way to define handedness is using questionnaires such as the Edinburgh Handedness Inventory[8] or the Waterloo Handedness Questionnaire[9], or a finger-tapping test[10]. In such questionnaires, a handedness index is calculated based on the self-reported handedness in a list of common manual tasks. However, the nature of handedness is so far unclear. An influential genetic model proposed by McManus[11] suggests that hand preference is controlled by an allele, which can be either right-biased (the D variant) or not biased (the C variant). Individuals with DD alleles are assumed to be right-handed; the handedness of individuals with CC alleles is random; and those with DC alleles have a 75% chance of right-handedness. A good fit of the data is obtained when the proportion of the C variant in the population is estimated to be around 0.155. However, although a few candidate genes have been proposed, and recent twin studies have confirmed a significant genetic influence on handedness, the genetic effects are complex and small, which suggests a polygenic control of handedness rather than a single-gene model[12].

The relationship between cerebral lateralization of language and handedness has been studied for years, and the link seems to be weak and indirect. On the other hand, left-handers are excluded from most cognitive studies in order to reduce variance in the data. Recently, a few studies suggested a weak but clear relationship between these two lateralized functions at the population level. For example, Knecht *et al.*[13] found that the likelihood of RH language dominance as measured with a word-generation task increases with the degree of left-handedness: ~1–5% of right-handed individuals are right-lateralized for language, and so are ~10–25% of left-handers. Given that LH language dominance cannot be generalized to the whole population, cognitive studies should take into account both left-handed and right-handed subjects[14]. Not only looking at typically organized cortices but also investigating atypical lateralization can help to unravel cortical organization. A shift in the hemispheric specialization of one functional network can reveal how another network is associated with or dissociated from the first. Language might be an interesting first network to look at, because RH speech dominance is rare but can be found in healthy people, especially in left-handed individuals, and this inspired the series of studies outlined below.

Is Language Dominance Related to Other Cognitive Functions?

Based on the report by Knecht *et al.*[13], Van der Haegen *et al.*[15] carried out large-scale screening of 265 left-

handers and determined their language dominance. The left-handers first took part in a behavioral visual half-field experiment, and were classified as atypical RH- or typical LH-dominant for speech if they were fastest at naming pictures and words presented on the left or right half of the screen, respectively (because of the partial crossing of optic fibers visual information is sent to the contralateral hemisphere). Their speech lateralization was confirmed in an fMRI word-generation task, in which activity in the inferior frontal gyrus was compared between the left and right hemispheres.

Then the left and right speech-dominant subjects took part in a study on reading lateralization. This made it possible to determine whether reading is dominantly processed in the LH because of low-level processes such as visual spatial frequencies[16,17] or because language sub-processes co-lateralize in order to optimize the integration of visual and phonological information. The lateralization indices based on activity in the ventral occipito-temporal (vOT – also termed the visual word form area[18] as it responds to (pseudo)words invariantly of retinal location, case, or font) during lexical decision showed that right dominance for speech in frontal language regions is most often accompanied by right lateralization of word recognition[19,20]. These results thus lend strong support to the hypothesis that vOT activity in word reading is adjusted 'top-down' by anterior language structures, instead of being automatically activated in a 'bottom-up' way. In other words, the vOT visual word recognition system is primarily a language system and not a visual processing system.

Although (a)typical functional lateralization can provide information on how language sub-processes interact, language does not exist in isolation from other cognitive functions such as memory and attention. For instance, a network has been shown to respond to different kinds of cognitive challenges[21]. This network, sometimes referred to as the 'cognitive control network' or 'multi-demand system', involves a set of regions in the prefrontal and parietal cortex, including dorsolateral prefrontal regions (cortex in and around the posterior part of the inferior frontal sulcus), anterior insular and adjacent frontal operculum, pre-supplementary motor area and adjacent dorsal anterior cingulate cortex, and regions in and around the intraparietal sulcus. Recent studies have investigated whether and how this domain-general cognitive control network is engaged in language processing, and have shown that cognitive control plays an important role in language, at least in language production (e.g. using a missing-letter paradigm[22] or verbal fluency[23]). In contrast, other studies (e.g. using a sentence understanding task[24]) found little or no response in language regions to non-language cognitive control. However, given that the regions involved in these functions are located in close proximity, especially in the prefrontal cortex, it is not easy to clearly separate them and draw conclusions. Again, this issue can be investigated *via* functional lateralization as an alternative approach. The research group that identified the (a)typical speech-dominant group noted above also examined the relationship between language production and non-language cognitive control, and found that cognitive control in a non-language task-switching paradigm is highly co-lateralized to the dominant hemisphere for language production (Fig. 1B, Cai *et al.*, unpublished data), which might indicate that the two functions share mechanisms. Apart from language production, visuospatial attention is the most salient lateralized cerebral function. Complementary specialization of language and visuospatial attention has been observed in the majority of the population. Does this complementary specialization have a causal origin (the lateralization of one function causes the opposite lateralization of the other for best parallel performance, as proposed by Kosslyn[25]), or is it rather a statistical phenomenon (different functions lateralize independently)? By testing the two groups of left-handers with opposite hemispheric dominance, Cai *et al.*[26] reported that right dominance of language is always accompanied by an atypical left-lateralized fronto-parietal network underlying visuospatial processing during a landmark task, both at the group and at the individual levels (Fig. 1A). These results clearly support the 'causal origin' hypothesis of complementary specialization, and we could speculate that this crossed lateralization has a longstanding evolutionary origin.

Furthermore, it has been reported that language and praxis (i.e., tool use) networks are highly overlapping and co-lateralize to the dominant hemisphere for language. This overlapping network involves the dorsolateral prefrontal cortex, posterior parietal cortex, supplementary motor area, and dorsal and ventral premotor cortex[27] (Fig. 1C).

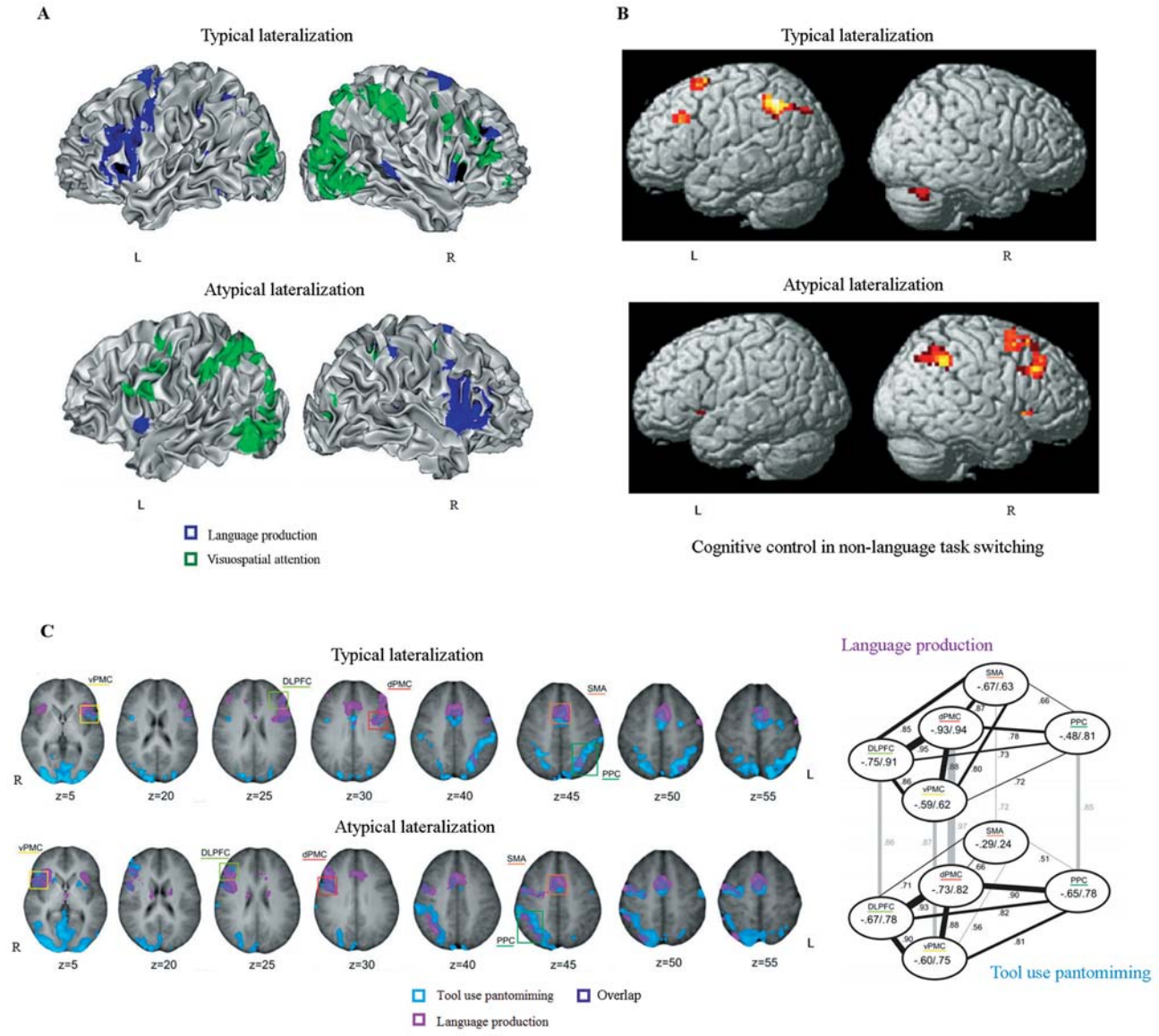

Fig. 1. (A) Language production and visuospatial attention lateralize to different hemispheres, independent of the side of lateralization; overlapping activations for the two tasks only occur in the insula and the supplementary motor area (SMA). (B) Cognitive control in a non-language task-switching paradigm is lateralized to the dominant hemisphere for language production, independent of the side. (C) Left panel: Language production and tool-use pantomiming co-lateralize to the same hemisphere, with overlapping activations (depicted in purple marked out by squares) in dorsolateral prefrontal cortex (DLPFC), dorsal premotor cortex (dPMC), ventral premotor cortex (vPMC), posterior parietal cortex (PPC) and SMA. Right panel: Co-lateralization within and between paradigms. The lateralization index (LI) of each region is listed inside the ellipses (typical/atypical lateralization group); black and gray connecting lines represent significant correlations between the LIs of the regions within and between paradigms, correspondingly. Note that these studies were conducted in left-handers. The figure is reproduced from Cai *et al*., Proc Natl Acad Sci U S A 2013[26] and Vingerhoets *et al*., Cortex 2013[27].

Both the direction and degree of lateralization during word generation correlate with the lateralization pattern during tool-use pantomiming. Most participants were left-handed, but the same pattern was found in one right-handed and one ambidextrous person. This indicates that handedness can only serve as an indirect selection criterion for models

linking gestures and speech to explain the evolution of human language[28]. Rather, the functional asymmetry of language or tool use can give new insights in this domain.

To conclude, functional lateralization studies seem to offer a different approach to investigate the relationship between different functions. Co-lateralization of different cognitive functions, or the dependency of their functional lateralization (i.e. complementary specialization), may suggest an interaction between the functions of interest, either online or during evolution/development. These studies could therefore add more evidence to our current research from a different point of view.

It should be noted, however, that these studies have so far been limited to pre-selected left-handers. Therefore, further studies are expected to confirm whether this conclusion can be generalized to the whole population, including both left- and right-handers. We should also note that many tasks widely used in current studies are not defined precisely enough, in the sense that they often involve cognitive functions other than the one of interest, such as memory retrieval, attention, and decision-making. Besides, a cognitive functional system, no matter which one, should not be considered as a whole, but rather a set of primitives (i.e. a 'parts list' of representational elements, as well as a list of elementary functions, from both the cognitive side and the neuroscience side[29]). Knowing how distinct parts of a cognitive function co-lateralize within an individual offers much richer and more detailed information about the mechanism underlying this cognitive function.

Asymmetries in the Human Brain and in Our Primate Relatives

Although the hemispheric lateralization of language is a specific cortical feature of the human brain, it is now clear that asymmetries of brain and behavior exist not only in humans but also in vertebrates and invertebrates[30-33]. Some of the asymmetries in animals parallel those in humans, probably serving as evolutionary precursors. It would therefore be unjust to argue that functional (language) lateralization studies in humans are the single best way to investigate cortical organization. Unique information for brain research can also be obtained by linking functional lateralization to the anatomical structure it is based in and by looking at the evolution of functions.

Chimpanzees, our closest relatives, show both a bias towards right-handedness at the population-level[30] and brain structural asymmetries in regions homologous with human language-relevant regions[31]. Furthermore, the direction of hand preference for clapping explains a significant portion of the variability in asymmetries of the planum temporale and inferior frontal gyrus[30]. In contrast, no significant population-level cerebral structural asymmetries have been reported in the macaque, except for the surface area of the superior temporal sulcus[31]. The asymmetries in chimpanzees are therefore suspected to be a precursor of human language lateralization.

A recent work by Leroy *et al*.[34] pointed out a robust human-unique asymmetry in the depth of the superior temporal sulcus (STS), which is deeper in the right than the left hemisphere. This asymmetry is systematically present in humans at all ages, but hardly detectable in chimpanzees and absent in macaques. Given that the STS region plays a crucial role in human linguistic functions, this asymmetry is suspected to be the spot underlying language lateralization. Nevertheless, the same study compared individuals with LH dominance for language and those with RH dominance, and found no significant difference in STS asymmetry between the two groups — they both showed a deeper STS on the right side. That is, this human-unique asymmetry seems not to be correlated with the functional lateralization of language. The morphometric results from the same two populations also showed that functional lateralization is only subtly linked to anatomical asymmetry, with differences in the surface area of the insula, part of the planum temporale, and the vOT[35]. Similarly, a leftward asymmetry in the relative fiber density of the arcuate fasciculus – connecting frontal and temporo-parietal language areas – was found for left- and right-handers irrespective of their functional lateralization during verb generation[36]. One study recently did find a relationship between the gyrification pattern of Heschl's gyrus involved in primary auditory processing and functional asymmetries during word listening, again irrespective of handedness[37]. To conclude, only subtle anatomical asymmetries have been linked so far to clear (a)typical functional language lateralization (note that other studies did relate the degree of only leftward lateralization to anatomical variations, e.g. [38]).

Towards an Ideal Theory of Hemispheric Specialization for Different Functions

Studies on functional lateralization in recent years have already shed light on the relationship between different cognitive functions. Nevertheless, the nature of hemispheric specialization is still far from clear.

For further research, an ideal theory of cerebral functional lateralization is expected to fulfill these requirements: (1) to include as many lateralized modules/functions as possible and take into account their co-lateralization; (2) to amplify research on the lateralization of functions other than speech so that they can serve as a starting point for lateralization research (i.e. comparing lateralization of sub-processes to the main function, charting the prevalence of (a)typical lateralization, and creating behavioral screening tasks to identify (a)typical subjects); (3) to better define distinctive (sub-)function systems involved in different cognitive functions, both theoretically and computationally, so that overlapping brain regions and networks can be optimally interpreted; (4) to clarify the link between functional lateralization and anatomical asymmetries including morphometric asymmetries and asymmetries in fiber pathways; (5) to take into account both left-handed and right-handed populations to be able to explain the probability and mechanism of atypical lateralization and handedness in some individuals; and (6) to associate the human model with human diseases, animal models, and genetic models.

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·Review·

Prefrontal cortical α_{2A}-adrenoceptors and a possible primate model of attention deficit and hyperactivity disorder

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Attention deficit and hyperactivity disorder (ADHD), a prevalent syndrome in children worldwide, is characterized by impulsivity, inappropriate inattention, and/or hyperactivity. It seriously afflicts cognitive development in childhood, and may lead to chronic under-achievement, academic failure, problematic peer relationships, and low self-esteem. There are at least three challenges for the treatment of ADHD. First, the neurobiological bases of its symptoms are still not clear. Second, the commonly prescribed medications, most showing short-term therapeutic efficacy but with a high risk of serious side-effects, are mainly based on a dopamine mechanism. Third, more novel and efficient animal models, especially in nonhuman primates, are required to accelerate the development of new medications. In this article, we review research progress in the related fields, focusing on our previous studies showing that blockade of prefrontal cortical α_{2A}-adrenoceptors in monkeys produces almost all the typical behavioral symptoms of ADHD.

Keywords: prefrontal cortex; α_{2A}-adrenoceptors; cognitive functions; attention deficit and hyperactivity disorder; animal models

Introduction

Attention deficit and hyperactivity disorder (ADHD) is one of the most prevalent childhood neurodevelopmental conditions, affecting 3–5% of grade-school children worldwide[1]. It is characterized by inappropriate levels of inattention, impulsivity, and/or hyperactivity[2-4]. These symptoms develop in childhood, and can persist into adolescence and adulthood[5]. ADHD seriously affects cognitive development[6-8], and, without appropriate treatment, has consequences for the risk of anxiety, substance abuse, and depression in adulthood[2, 5, 9].

The neurobiological bases of ADHD symptoms are still not clear[10]. Clarifying them can help better understand the biological vulnerabilities that may underlie ADHD in a specific patient and how to modulate the responses to treatment, thereby contributing to better and more effective therapy.

It has been suggested that the symptoms involve a dopaminergic mechanism in the prefrontal cortex (PFC) and striatum[11, 12]. Experimentally decreased dopamine (DA) release in the PFC results in ADHD-like symptoms[13, 14]. To date, DA dysregulation is thought to be central to the neurobiology of ADHD, and its pharmacological treatment, such as methylphenidate (MPH, i.e. Ritalin)[15-17], levels the DA concentration in the synapse and extrasynaptic space in the PFC as a blocker of the DA transporter. MPH ameliorates inappropriate inattention[18-20], decreases impulsivity[21], and enhances inhibitory control[22]. However, as MPH is a prescription psychostimulant, there are strong concerns over drug dependence, paranoia, schizophrenia, and behavioral sensitization that might be caused by long-term therapy, similar to other stimulants[23-25].

Converging evidence indicates that the patho-physiology of ADHD has multiple origins[26-32]; for instance, norepinephrine (NE) has long been implicated[33, 34]. In this paper, we review research progress in the relevant fields, focusing on the potential relationship between prefrontal α_{2A}-adrenoceptors and ADHD in nonhuman primates[35-39].

Prefrontal Cognitive Dysfunctions in ADHD

The PFC plays a key role in cognitive functions such as working memory, the regulation of attention, and behavioral inhibition. Imaging and neuropsychological studies have shown that patients with ADHD have poor PFC functions, including poor attention regulation[40], limited working memory[41], and inability to inhibit inappropriate motor activity[42].

Working memory is a fundamental higher-order function, underlies a wide range of executive functional processes[43, 44], and is primarily controlled by the PFC[45, 46]. It has been shown that ADHD patients have altered architecture and less activation in the PFC[47-49]. Persistent working-memory problems are the main cognitive deficit in ADHD[40, 41, 50, 51].

Attention brings sensory or mental stimuli to the forefront of awareness[40, 52], and plays a pivotal role in mediating the executive functions of the PFC. During distracted states, the capacity to diminish the awareness of relevant stimuli is compromised. Compared to normal peers, ADHD patients show attention deficits in detecting invalidly-cued targets with slower speed and less accuracy[53, 54].

Inhibitory control of behavior is one of the most important functions of the PFC[55]; ablation or lesion of the frontal cortex in monkeys induces locomotor hyperactivity[56-58]. Perhaps the most fundamental deficit in ADHD is the lack of response inhibition[52]. In laboratory studies of tasks that measure inhibitory control, children with ADHD often perform more poorly than both normal controls and children with other psychiatric disorders[59, 60]. Schulz *et al.* reported that response inhibition in adolescents diagnosed with ADHD is primarily mediated by fronto-striatal circuitry[61, 62].

Prefrontal α_{2A}-Adrenoceptor Blockade Produces ADHD Phenotypes

ADHD has been posited to be caused by hypofunctional catecholamine systems[63] in multiple brain regions including the PFC[64-66] and striatum[67]. Implicated in this are NE projections that originate primarily from neurons in the locus coeruleus and send projections to multiple regions, including the PFC[68]. There are many subtypes of adrenergic receptors in the PFC, including the α_{2A} subtype. The α_{2A}-adrenoceptors are localized at both pre- and post-synaptic NE terminals[69]. However, studies in rodents, monkeys, and humans have shown that lower to moderate levels of NE have a beneficial influence on prefrontal cognitive functions through action at post-synaptic α_{2A}-adrenoceptors[64, 70].

ADHD symptoms can be mimicked by blockade of α_{2A}-adrenoceptors in the PFC. To investigate the role of prefrontal α_{2A}-adrenoceptors in the inhibitory control of behavior, we trained two monkeys to perform a go/no-go task, and the α_{2A}-adrenergic antagonist yohimbine was infused bilaterally and chronically into the dorsolateral PFC with mini-osmotic pumps. We found that blockade of the α_{2A}-adrenoceptors selectively impaired the “no-go” performance of monkeys, leaving the “go” performance intact. In quite a few cases, the monkeys should have kept their hands still and not touch the screen (no-go), but they made a response to the screen[38]. Infusion of saline at the same cortical locations did not affect the no-go performance, indicating that the yohimbine-induced impulse was not because of nonspecific factors such as infusion-induced cortical damage (Fig. 1A). Our previous work provided the first behavioral evidence that α_{2A}-adrenocepters in the dorsolateral PFC are involved in the inhibitory control of behavior.

In addition, the monkeys’ locomotor activity was monitored before, during and after yohimbine infusion into the dorsolateral PFC. Compared to that before administration, the daily locomotor activity increased dramatically during the 8-day administration of yohimbine; this gradually returned to normal after the infusion was stopped (Fig. 1B). Infusion of saline at the same location did not cause locomotor hyperactivity[39]. This work suggests that the α_{2A}-adrenoceptors in the dorsolateral PFC are associated with locomotor activity, and the dorsolateral PFC dysfunction of α_{2A}-adrenergic transmission could be one of the main causes of the impulsive behaviors and hyperactivity in children with ADHD.

Due to the limitations of working on nonhuman primates, we also implemented similar experiments on rats to assess the dose-dependent and age effects of yohimbine at a homological cortical site, the medial PFC. The results showed that yohimbine infused into the medial PFC dose-dependently induced hyperactivity in rats of different ages, and the trends showed that the younger the rats, the more hyperactivity

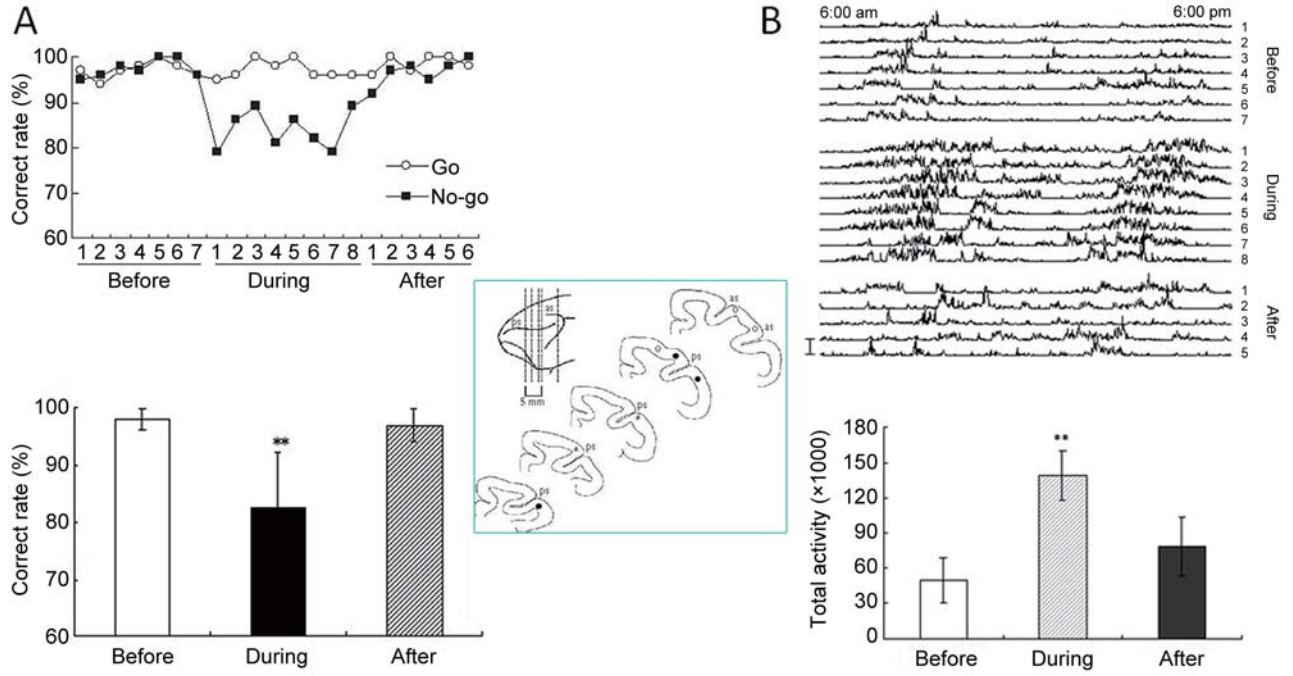

Fig. 1. Yohimbine infused bilaterally and chronically into the dorsolateral prefrontal cortex impairs impulse control and induces locomotor hyperactivity (adapted from Ma CL *et al.,* Neuroreport, 2003[38] and Biol Psychiatry, 2005[39]). (A) Yohimbine impairs "no-go" performance but has no effect on "go" performance. In several cases, the monkeys should not touch the screen (no-go), but they make a response. (B) Daily locomotor activity increases during administration of yohimbine. Each trace is a daily recording from 06:00 to 18:00. Inset: Reconstructed sites for chronic administration of yohimbine and saline. Filled symbols, yohimbine infusion; open symbols, saline infusion; as, arcuate sulcus; ps, principal sulcus.

presented at the same dose (unpublished data). All these results showed that dysfunction of the PFC α_{2A}-adrenoceptors results in the behavioral problems seen in ADHD.

ADHD symptoms can also be induced in humans by reducing the stimulation of α_{2A}-adrenoceptors. Kopeckova *et al.* investigated a polymorphism in the promoter region of the gene encoding DA beta-hydroxylase, an enzyme that reduces NE synthesis, and found that the affected children had poor sustained attention, weaker impulse control, and impaired executive function[71]. Genetic alterations in α_{2A}-adrenoceptors also impair PFC executive function, and lead to conditions seen in ADHD[72]. Thus, prefrontal α_{2A}-adrenoceptors are required for attention and behaviors in humans too.

Prefrontal α_{2A}-Adrenoceptor Stimulation Ameliorates Cognitive Dysfunctions in ADHD

Behavioral, pharmacological, and electrophysiological research has shown that stimulation of α_{2A}-adrenoceptors has a beneficial influence on PFC cognitive functions. Arnsten *et al.* found that systemic administration of the α_{2A}-adrenergic agonist guanfacine improves working memory in monkeys[73]. Steere demonstrated that systemic administration of guanfacine improves visual object discrimination reversal performance in aged rhesus monkeys[74]. Our work showed that both systemic administration and local infusion of guanfacine into the PFC improve visuomotor associative learning[70, 75]. Using an iontophoretic technique, stimulation of α_{2A}-adrenoceptors in the PFC was found to increase the spiking activity associated with working memory in behaving monkeys[37, 76].

Neuronal activity in the PFC associated with working memory can be enhanced by α_{2A}-adrenoceptor stimulation through cAMP-HCN signaling pathways[76, 77]. Our work suggested that under normal physiological conditions, the α_{2A}-adrenoceptors in pyramidal cells can be activated through Gi-cAMP-HCN signaling[78]. On the other hand,

under stress, activation of α_{2A}-adrenoceptors to protect PFC functions might occur *via* the Gi-cAMP-PKA-CaMKII-AMPAR signaling pathway[76]. Both mechanisms together optimize the synaptic inputs to pyramidal neurons and determine the synaptic outputs for PFC cognitive functions. Indeed, it has been reported that guanfacine at relatively high doses suppresses evoked excitatory postsynaptic currents, and has no enhanced effect or even suppresses delay-related activity[76].

New Insights to Develop a Primate Model of ADHD

As noted above, most of the commonly-prescribed medications for ADHD are psychostimulants, which are reported to have short-term therapeutic efficacy but with a high risk of serious adverse effects with long-term treatment. It is urgent to find new medications with high therapeutic efficacy and low adverse effects for children with ADHD. The first step now is to develop novel animal models of ADHD. A good model should very nearly show the fundamental behavioral characteristics of ADHD, conform to a theoretical rationale for ADHD, account for the neurobiology, and respond to therapeutic interventions both behaviorally and pharmacologically[79].

Currently, animal models of ADHD are genetic and non-genetic[80]. The spontaneously-hypertensive rat (SHR), the most widely used model, is a genetic model[81, 82]. SHRs exhibit hyperactivity[83, 84], impulsivity/inattention[82, 85], and poor learning and memory[86]. They also have disturbances in glutamate, DA, and NE functions, which in parallel demonstrate that ADHD patients have defects in the neuronal circuits required for reward-guided associative learning and memory formation[87]. Clearly, the SHR is a good model for the study of memory deficits in ADHD, primarily in the context of particular risk factors/symptoms, responsiveness to specific drugs or other treatments or biomarkers for the diagnosis of ADHD, and for understanding the pathological mechanisms for the development of therapeutic approaches. However, SHRs do not fulfill all the behavioral and pharmacological profiles of an ADHD model; for example, ADHD-like behaviors in SHRs are not restricted to males[88]. Hyperactive behavior in SHRs is ameliorated only by high doses of amphetamine or MPH[84], unlike ADHD patients, whose behavioral deficits can be improved with low doses of MPH. Importantly, ADHD patients show reduced regional cerebral blood flow in the frontal cortex[89], while SHRs do not[90,91].

Our previous research with monkeys indicates that blockade of the prefrontal α_{2A} receptors induces locomotor hyperactivity, impulsivity, and poor attention regulation/working memory. These results verify the feasibility and acceptability of treating ADHD by stimulating α_{2A}-adrenoceptors in the PFC or up-regulating the NE concentration in synapses and extrasynaptic space in the PFC. Actually, the α_{2A}-adrenergic agonists guanfacine and clonidine have been used experimentally and clinically to treat ADHD[92-97]. The selective inhibitor of the NE transporter atomoxetine (tomoxetine or LY139603) has also been reported to alleviate ADHD symptoms[98]. All these medications have achieved much better therapeutic efficacy with less adverse effects[99] than MPH and amphetamines, although there is controversy regarding the long-term effectiveness[100, 101].

Thus, in the future, studies should focus on developing a novel ADHD model in nonhuman primates, by down-regulating or blocking the α_{2A}-adrenoceptors in the dorsolateral PFC. This could be realized by chronic bilateral infusion of yohimbine. This kind of animal model could approximate the fundamental behavioral characteristics of ADHD, conform to a theoretical rationale for ADHD associated with prefrontal α_{2A}-adrenoceptors, and account for the neurobiology and therapeutic interventions in terms of both pharmacological and behavioral functions.

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·Review·

Prefrontal dysfunction and a monkey model of schizophrenia

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The prefrontal cortex is implicated in cognitive functioning and schizophrenia. Prefrontal dysfunction is closely associated with the symptoms of schizophrenia. In addition to the features typical of schizophrenia, patients also present with aspects of cognitive disorders. Based on these relationships, a monkey model mimicking the cognitive symptoms of schizophrenia has been made using treatment with the non-specific competitive N-methyl-*D*-aspartate receptor antagonist, phencyclidine. The symptoms are ameliorated by atypical antipsychotic drugs such as clozapine. The beneficial effects of clozapine on behavioral impairment might be a specific indicator of schizophrenia-related cognitive impairment.

Keywords: prefrontal cortex; schizophrenia; monkey model

Introduction

One percent of the adult population, particularly young adults, are affected by schizophrenia. Schizophrenic patients typically present with hallucinations, delusions, withdrawal from social activities, loss of personal care skills, and flat or inappropriate emotional responses to situations[1]. Although many factors have been associated with schizophrenia, including genetic factors, early environmental influences, and neurobiological, psychological, and sociological processes[2-4] that are very important contributory factors, the mechanism underlying this disorder is unknown. Patient data are insufficient for understanding the pathology and etiology of schizophrenia. Further, direct experimentation on human subjects is ethically unacceptable. Thus, animal models have become an indispensable tool for pathological research. Unlike other neurological diseases such as stroke, epilepsy, and Parkinson's disease that can be easily replicated in animals, some of the symptoms of schizophrenia human patients, such as thinking disorders, delusions, and hallucinations, are difficult to replicate in animals. For many years, there has been no appropriate non-human primate model of schizophrenia. In addition to the typical features of schizophrenia, patients present with aspects of prefrontal cognitive disorders involving, e.g., working memory, selective attention, initiating movement, and planning. Based on these relationships, a monkey model of schizophrenia could be developed by mimicking its cognitive symptoms. In this review, we introduce schizophrenia and the prefrontal cortex (PFC) as well as their relationship and discuss the development of a monkey model of schizophrenia, including the methods and behavioral tests of the model.

The Prefrontal Cortex and Schizophrenia

The volume of the human brain is twice that of the chimpanzee, and the PFC, which is the most rostral component of the primate cortex, has expanded most in humans[5,6]. In other species, the PFC functions in voluntary motor control, whereas in primates, it has developed

significantly to include other important functions such as executive functions, conflicting-thought mediation, decision-making (e.g., distinctions of right *versus* wrong or good *versus* bad), prediction of future events, and government of social and emotional control. Further, the PFC in the human brain plays a central role in conscience, intelligence, and personality[5-7]. The PFC has broad connections with many parts of the brain, particularly the sub-regions of the limbic system[10]. The human PFC, which is better developed than in other primates, is the primary contributor to humans having unequalled abilities for planning and abstract reasoning[6,8].

Prefrontal dysfunction plays a role in the expression of the cognitive deficits associated with schizophrenia[9]. The relationship among the PFC, executive dysfunction, and schizophrenia symptoms has been studied, and several studies have shown the existence of abnormalities in the PFC. MRI studies have revealed a decrease in the volume of PFC in schizophrenic patients[10-13], and a postmortem anatomical study found that the cortex of prefrontal area 46 is thinner and the density of neurons in this area is increased[14-16]. In as early as 1986, to study the relationship between prefrontal dysfunction and schizophrenia, medication-free chronic schizophrenia patients and normal controls were used, and the regional cerebral blood flow (rCBF) was measured during performance of a PFC-specific cognitive test, the Wisconsin Card Sorting (WCS) or a simple number-matching (NM) test as the control. During the WCS, there was a clear increase in PFC rCBF in the controls compared with the schizophrenic patients; the PFC was the only region that changed. This finding significantly distinguished patients from controls, whereas during the NM, no region differentiated patients from controls. Further, the PFC rCBF was positively associated with WCS performance in patients, suggesting that the better the PFC functions, the better patients perform[9]. The decreased prefrontal rCBF in schizophrenic patients might be a manifestation of abnormal prefrontal neuronal activity. In addition, considerable effort has been directed at identifying specific neuronal factors that contribute to PFC dysfunction in schizophrenic patients. These findings are consistent with the hypothesis that schizophrenia is associated with complex alterations in the anatomy and function of multiple neuronal populations in the PFC. At the cellular and molecular levels, chronic stress exposure leads to spine loss in the PFC[17,18], particularly in layer III, which harbors the recurrent microcircuits that are most affected in schizophrenia[19]; the interconnection of precise microcircuits in the dorsolateral PFC appears to be associated with schizophrenia. Physiological studies in monkeys indicate that recurrent excitation between PFC pyramidal cells depends on the N-methyl-*D*-aspartate (NMDA) receptors, and several genes associated with NMDA transmission have been linked to schizophrenia [20].

The monoamine system, particularly the dopamine (DA) system, is important for prefrontal cognitive function, so many PFC functions are related to the DA system[21]. Dysfunction of the prefrontal monoamine system is associated with mental disorders such as schizophrenia, attention-deficit/hyperactivity disorder, drug addiction, autism, and depression[22-28]. Neurochemical analyses of DA and its metabolites provide evidence that mesoprefrontal DA neurons play a role in prefrontal dysfunction in schizophrenia and that the extent of decrease of DA turnover is directly associated with the severity of cognitive deficits[29-32]. In addition, the density of D1-like receptors in the PFC is decreased in schizophrenic patients who show prefrontal cognitive impairments[32]. On the contrary, DA agonists improve cognitive performance and cognitive activation of blood flow in the PFC of schizophrenic patients, which supports the hypothesis that hypofunction of the mesoprefrontal DA neurons contributes to schizophrenia[21].

Based on these facts, Goldman-Rakic and some scientists suggested that prefrontal dysfunction, particularly the impairment of working memory, is a cause of schizophrenia[14-16, 28-32, 50]. This suggestion led to the development of a novel monkey model of schizophrenia that mimics the cognitive symptoms of schizophrenia.

The Monkey Model of Schizophrenia

Based on this idea, in 1997, Jentsch *et al.* reported the development of a monkey model of schizophrenia using phencyclidine (PCP), a non-specific competitive NMDA receptor antagonist, in which the monkeys exhibit task performance deficits involving prefrontal cognitive function after PCP treatment twice a day for two weeks. In addition, they found that clozapine, an atypical antipsychotic drug, ameliorated the performance deficits. This study showed that the repetition of PCP treatment in monkeys might be

an effective method of studying psychiatric disorders such as schizophrenia, which involves cognitive dysfunction and DA hypo-function in the PFC[33].

Drugs for Developing an Animal Model of Schizophrenia

Three drugs are used for the development of a monkey model of schizophrenia, ketamine, PCP, and MK801. Here, we focus on ketamine and PCP.

Ketamine is an NMDA receptor antagonist that is predominantly used for the induction and maintenance of general anesthesia. Long-term treatment with ketamine can cause a number of impairments in cognitive function. The effects of ketamine are brief, and last no more than a few hours; its hallucinatory effects last ~1 h. At sub-anesthetic doses, ketamine induces a dissociative state[34-38].

PCP is also an NMDA receptor antagonist. It is a synthetic dissociative drug originally developed as a general anesthetic. It is a partial agonist of dopamine D2 receptors, which might explain the psychotic symptoms caused by PCP. In humans, PCP can cause schizophrenia-like symptoms, and it has become a useful tool for developing animal models of this disease[39-43]. The short-term psychotic effects include: visual and auditory hallucinations; feelings of unreality and dissociation from the environment; distorted sense of body and time and space; distorted thinking; anxiety; paranoid thoughts; confusion and disorientation; intense feelings of alienation; depression; bizarre or hostile behavior; and grandiose delusions. The effects of long-term use are the following: chronic and severe anxiety and depression; social withdrawal and isolation; impaired memory; and persistent speech problems, such as stuttering. In monkeys, it is difficult to detect hallucinations with the PCP treatment. The effects of PCP in nonhuman primates include impairments in working memory and motor programming, as well as behavioral inhibition.

Possible Genetic Monkey Models of Schizophrenia in the Near Future

Besides drug models, studies involving twins have shown that schizophrenia is a heritable disorder and several schizophrenia-related genes have been revealed. For example, disrupted in schizophrenia 1 (*DISC1*) was one of the first genes discovered to be involved in schizophrenia. Anatomically, DISC1 knockin mice show increased lateral ventricle size, reduced cortical and hippocampal size like those found in schizophrenic patients[51]. Also, the expression of several proteins changes in the brain of schizophrenic patients such as “dysbindin” encoded by the gene *DTNBP1*, which is thought to be one of the most promising candidate genes for schizophrenia[52]. Another gene is *NRG1* which codes for a growth factor crucial to the development of the nervous system. Partial knockout of NRG1 in mice causes social interaction problems, reduced prepulse inhibition, and higher levels of spontaneous locomotion. These symptoms are reduced by clozapine[51].

It used to be quite difficult to perform genetic manipulation in monkeys, but at present, these techniques are rapidly developing in nonhuman primates[53,54]. Thus it will be possible to develop genetic monkey models for schizophrenia by genetic manipulation techniques in the near future.

Behavior Testing

The major prefrontal function is executive function, which includes planning and regulating, which are difficult to model in animals. Here, we describe two methods of detecting cognitive dysfunction in animal models.

The object retrieval detour (ORD) task The ORD task measures inhibitory control, which is a component of executive function. The main compartment of the test apparatus is put in front of the monkey. In the main compartment, there is a transparent plastic reward box that is open on one side. The open side of the box can be oriented directly toward, to the right side, or to the left side of the monkey, and the monkey can reach the box in all positions. This box can also be placed at the center or on the right or left side of the main compartment (Fig. 1). In this task, the monkey is required to retrieve food, which is placed in the reward box as a reward. The difficulty of the task performance is set up in the following three ways: the placement of the reward box within the main compartment, the orientation of the open side of the reward box, and the location of the food reward within the reward box (e.g., near or far from the opening). To perform this task, a monkey must inhibit the tendency to reach directly toward the food. To inhibit this tendency, several PFC functions are required. The motor performance in the ORD task is sensitive to impairments of the PFC and the dopamine system[44-49].

The delayed-response (DR) task DR tasks are normally used to investigate working memory processes. Providing

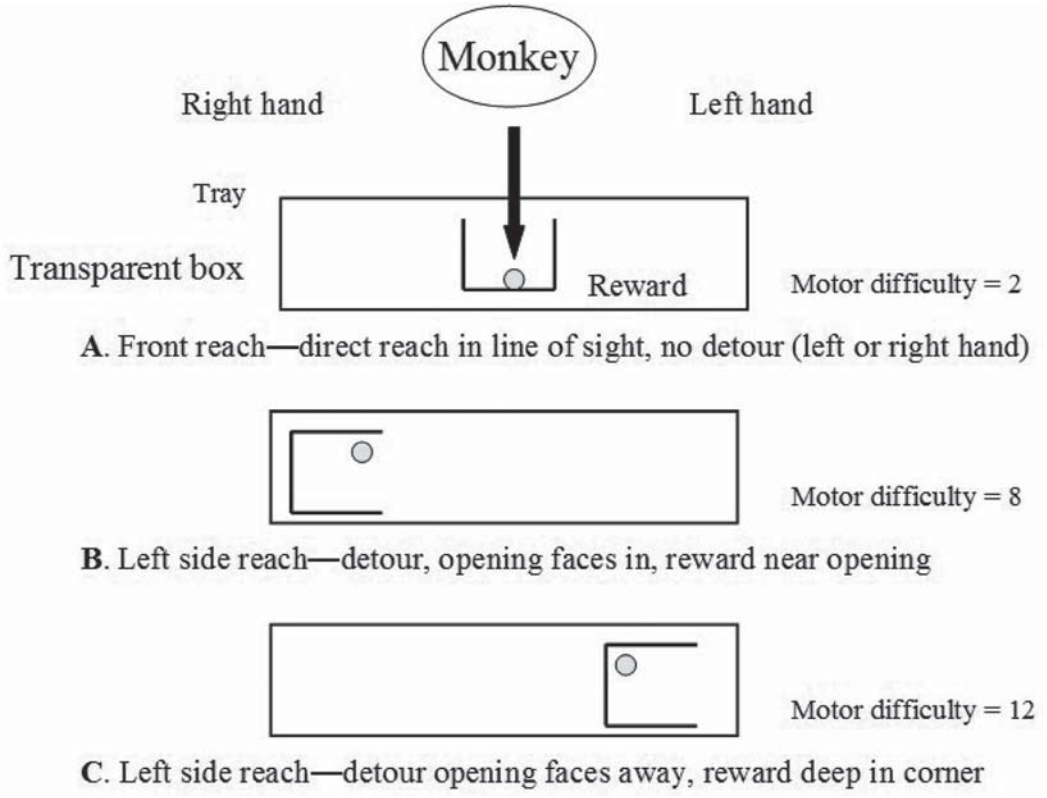

Fig. 1. The object retrieval detour task consists of attempting to retrieve a food reward placed in a transparent box that has one open side. The open side can be oriented toward, to the right, or to the left of the monkey[33].

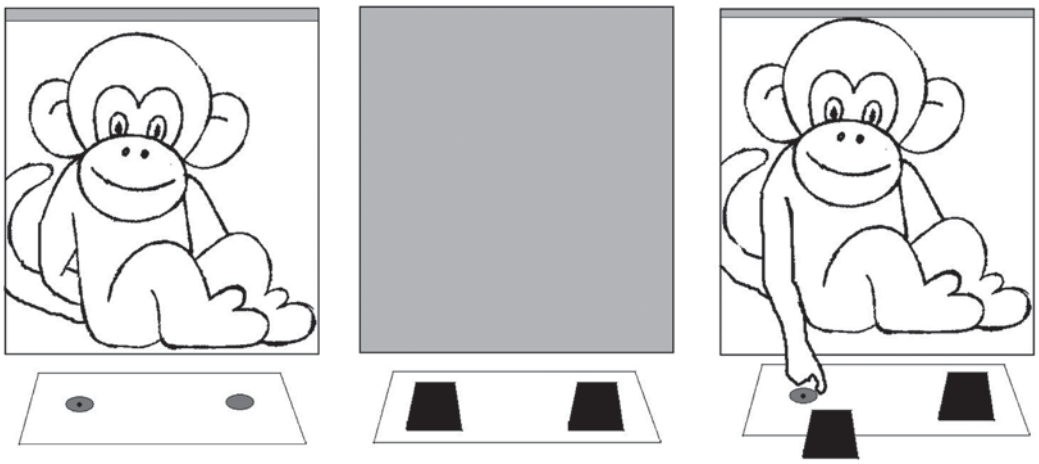

Fig. 2. In the delayed-response task, the monkey watches an experimenter place a food morsel in one of two wells (left); both wells are then covered. Subsequently, a screen is lowered for an interval of a few seconds to several minutes (the delay) (middle). When the screen is raised, the monkey gets only one chance to uncover the well containing food and receive the reward (right)[55].

different stimuli in different trials creates a task in which the test animal must remember varieties of the cue from trial to trial using working memory.

In a typical DR task, the monkey must choose from two wells, only one of which contains food (Fig. 2). With a delay (from a few seconds to several minutes) by lowering and raising a screen between the monkeys and the wells, the monkeys have only one chance to uncover the well to receive the reward after watching the placement of the food[9].

The repetition of PCP administration induces behavioral and biological impairments similar to those of schizophrenia. It has been reported that clozapine ameliorates the symptoms of schizophrenic patients refractory to treatment. Thus, clozapine would have an effect on the behavioral improvement of animals previously treated with PCP. The beneficial effects of clozapine on behavioral impairment might be a “specific indicator” of schizophrenia-related cognitive impairment.

Conclusion

The PFC is implicated in cognitive functioning and schizophrenia, and there is a close relationship between prefrontal dysfunction and the symptoms of schizophrenia. In addition to the typical features of schizophrenia, patients present with aspects of cognitive disorders. Based on these relationships, a monkey model of schizophrenia could be developed by mimicking the cognitive symptoms, which are ameliorated by atypical antipsychotic drugs such as clozapine. The beneficial effects of clozapine on behavioral impairment might be a “specific indicator” of schizophrenia-related cognitive impairment.

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·Research Highlight·

Learning to memorize: shedding new light on prefrontal functions

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Working memory is one of the essential higher cognitive functions that actively holds behaviorally-relevant information essential for guiding subsequent actions. It includes subsystems that store and manipulate single-mode or multi-modal sensory information, e.g., spatial information, visual images, auditory scenes, olfactory objects, or any combination of these. In addition to merely holding a certain amount of information for a short period of time, as is generally believed, the cognitive processes involved are far more complex, including the executive and attentional control of short-term memory, permitting interim integration, and the processing, disposal, and retrieval of information. Evolution-wise, working memory is essential for the behavioral flexibility that allows humans and animals to quickly adapt to rapidly changing environments.

A wealth of studies have been conducted in attempts to understand the neuronal process underlying working memory, and have identified a number of brain regions as crucial, including the prefrontal cortex (PFC), posterior parietal cortex, anterior cingulate, and parts of the basal ganglia. Among these regions, the PFC has drawn most attention due to the striking finding that individual neurons show persistent activity during the memory-retention period[1-3] (termed the delay period, a hallmark of working memory tasks): elevated activity persists after the sensory stimuli have been removed until the holding period is over (from seconds to tens of seconds) and the behavioral choice has been made. This raises the immediate question of whether the persistent activity in the PFC during the delay period encodes the contents of working memory (memory storage). This has been under debate for the last two decades[4]. Some studies find that PFC activity increases when the number of items to be memorized increases. This seems to support the hypothesis that the PFC plays an important role in memory storage, as a straightforward explanation would be that increasing the demands of storage would be expected to increase the activity level in a region where representations are being actively stored. However, an equally plausible explanation would be that if PFC activity reflects top-down signals to control more posterior regions where the actual representations are stored, maintaining higher loads of information might require increased PFC input in order for retained information to survive delay and distraction. Therefore, it is not yet clear that the PFC is the site where the representations are stored. The fact that the PFC has been found to play important roles in executive functions[4] implies that its role in working memory might be controlling attention, selecting strategies, and manipulating information, rather than information storage[2, 5].

To resolve this debate, it is therefore necessary to achieve a deeper understanding of the causal role of the PFC in working memory tasks. This would require temporally precise perturbation of neuronal activity in specific regions of the PFC during the delay period of a working memory task and monitoring its effect on task performance. Technically, such manipulation has not yet been achieved in primate and human subjects due to technical difficulties. In rodents, however, the temporally and spatially precise manipulation of neuronal activity has been exceedingly successful thanks to the recent development of new tools such as optogenetics and genetic manipulation techniques[6]. Meanwhile, choice-based and precisely controlled behavioral paradigms in rodents have also been developed, allowing a high degree of stimulus control, accurate behavioral readout, and precise measurement of neuronal activity[7–9]. On top of

these behavioral paradigms, imposing an additional delay period before choice allows precisely timed, memory-based brain processes to be investigated using rodents[10, 11]. However, imposing a delay immediately before choice could confound the memory content with motor planning components[10]. In order to determine whether PFC activity during the delay period is responsible for memory storage, a more desirable paradigm would try to retrieve the same sensory information following a delay period, such that decision or behavioral choice can be made only after the memory retrieval is finished.

In a recent study[12], using head-fixed mice, Liu *et al.* developed an olfactory delayed-nonmatch-to-sample paradigm (DNMS), a standard working memory task that had only been used in primates before (Fig. 1). In this task, head-fixed mice were presented briefly, at the beginning of each trial, with one of two odorants, and after a 4–5 s delay period, a second odorant was presented. The animal needed to decide whether the second odorant was the same as or different from the first one. If the two odorants differ, the animal should respond by licking ("go" response) a lickport, otherwise, the animal should withhold licking ("no-go" response). Therefore, the animal had to retain the information of the first odorant for the entire delay period in order to compare it with the second one: a typical requirement in working memory tasks. Mice can readily learn this memory-based decision task in as few as 5 days, and the learning process can be monitored.

This new paradigm in mice has opened up a playground for a range of manipulation and recording techniques such as optogenetic tools and multi-electrode recording for dissecting the functional role of the PFC in working memory. By expressing channelrhodopsin-2 (ChR2) in inhibitory interneurons or *Natronomonas* halorhodopsin in excitatory pyramidal neurons in the medial prefrontal cortex (mPFC), Liu *et al*. silenced the mPFC using light stimulation only during the delay period and examined the DNMS task performance. The findings are rather striking: the activity in the mPFC during the delay period is only required during the learning phase, typically from day 1 to day 5, but not for well-trained animals. This can shed light on the role of the mPFC in memory storage during the working memory task, if one considers the difference between the underlying processes in different learning stages: during the learning phase, many novel, attention-demanding components could occur during the delay period in order for the subject to accomplish the task, while after becoming well-trained, memory storage becomes the major if not the only factor that matters during the delay period. It is therefore suggested that the delay-period mPFC activity is necessary only for a novel, attention-demanding working memory task, but not for the simple short-term storage of olfactory information in the well-trained stage. This provides new clues to the lasting debate on whether the mPFC is the location for memory storage or rather for conducting executive functions such as controlling attention, selecting strategies, and manipulating information.

Another question concerns the specificity of the persistent activity in the mPFC during the delay period, i.e., whether it requires a specific subpopulation of neurons in the mPFC to be activated, or a general elevation of

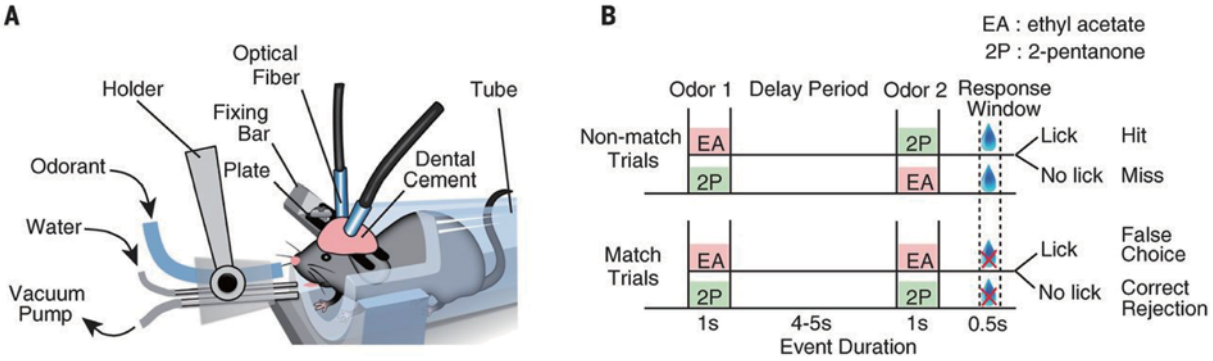

Fig. 1. Behavioral paradigm. A. Apparatus for head-fixed go/no-go paradigm using olfactory cues, compatible with optogenetic stimulation. B. Task structure for olfactory DNMS paradigm. Adapted from the reference[12] with permission.

mPFC activity is sufficient. Instead of silencing it, Liu *et al.* activated the mPFC during the delay-period by expressing ChR2 in excitatory neurons and delivered blue light only during the delay period, which led to a general elevation of mPFC activity. Interestingly, this manipulation impaired, rather than improved, the task performance during learning stages, but not in the well-trained stage. Therefore, the working memory task during the learning stages requires the activation of a rather specific subpopulation of mPFC neurons, although their exact specificity requires further investigation.

An important question regarding the persistent activity in the mPFC is whether and how it evolves with learning, which was rarely addressed in earlier studies. In the study by Liu *et al*., the authors monitored population activity in the mPFC throughout the course of learning a working memory task. Indeed, the population dynamics in the mPFC evolves with learning: the delay-period activity is more prominent and distinguishes the two odorant stimuli in the learning stages, but this diminishes in the well-trained stage, consistent with the optogenetic manipulation results. This provides another dimension of evidence that the mPFC is involved in the attention-demanding learning phase, rather than in a simple memory-storage process in the well-trained stage.

Liu *et al.* developed a standard working memory assay in mice, and combined it with temporally precise neuronal perturbation and recording techniques, from which the authors provided new evidence that could help resolve the long-standing debate over the functional role of the persistent prefrontal delay-period activity in working memory. It seems that at least part of the prefrontal region, the mPFC, is crucial for animals to accomplish novel, attention-demanding, and memory-based tasks, but is not required for memory storage *per se*. This suggests that future investigations should focus more on additional brain regions in memory storage in the working memory task; this storage may be distributed, involving multiple brain regions in the hierarchy, including the sensory areas of relevant modalities.

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·Original Article·

Single-nucleotide polymorphisms and haplotypes of non-coding area in the *CP* gene are correlated with Parkinson's disease

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ABSTRACT

Our previous studies have demonstrated that ceruloplasmin (CP) dysmetabolism is correlated with Parkinson's disease (PD). However, the causes of decreased serum CP levels in PD patients remain to be clarified. This study aimed to explore the potential association between genetic variants of the *CP* gene and PD. Clinical features, serum CP levels, and the *CP* gene (both promoter and coding regions) were analyzed in 60 PD patients and 50 controls. A luciferase reporter system was used to investigate the function of promoter single-nucleotide polymorphisms (SNPs). High-density comparative genomic hybridization microarrays were also used to detect large-scale copy-number variations in *CP* and an additional 47 genes involved in PD and/or copper/iron metabolism. The frequencies of eight SNPs (one intronic SNP and seven promoter SNPs of the *CP* gene) and their haplotypes were significantly different between PD patients, especially those with lowered serum CP levels, and controls. However, the luciferase reporter system revealed no significant effect of the risk haplotype on promoter activity of the *CP* gene. Neither these SNPs nor their haplotypes were correlated with the Hoehn and Yahr staging of PD. The results of this study suggest that common genetic variants of *CP* are associated with PD and further investigation is needed to explore their functions in PD.

Keywords: Parkinson's disease; ceruloplasmin; single-nucleotide polymorphism; haplotype; copy-number variation

INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra[1]. The presence of Lewy bodies in degenerating dopaminergic neurons seems to be the initial characterization of the pathology of PD. In the core of the Lewy body is the protein α-synuclein which binds iron. Iron seems to be a requisite for the deposition and accumulation of α-synuclein. Thus, iron might be deposited in a disorderly manner in extrapyramidal structures and result in the tissue damage in PD. However, little is known about the cause of iron deposition in PD.

Ceruloplasmin (CP) is a multi-copper enzyme with ferroxidase function and plays an important role in iron metabolism[2]. CP oxidizes ferrous iron into the ferric form and thus keeps the intracellular level of dangerous ferrous iron to a minimum[3, 4]. Hereditary aceruloplasminemia caused by mutation of the *CP* gene, resulting in the absence of circulating serum CP, presents with parkinsonism and retinal degeneration due to substantial iron accumulation in the basal ganglia and retina[5]. Patel and colleagues have also demonstrated that increased iron accumulation and free-radical injury occur in the central

nervous system of *Cp*$^{-/-}$ mice[6]. Thus, the association between CP and PD has been discussed since the ninth decade of the last century. It has been found that both the CP level and oxidative activity in serum and cerebrospinal fluid are significantly lower in PD patients than in age- and sex-matched healthy controls[7-13]. In addition, lower serum CP levels are correlated with a younger age of PD onset[14]. Furthermore, our previous studies and those of others have demonstrated that decreased serum CP level and oxidative activity specifically exacerbate nigral iron deposition in PD patients[15-17]. However, the causes of the decreased serum CP level in PD remain to be clarified.

As PD often has a hereditary basis and the genetic predisposition is seen as a major contributor to the underlying cause, we carried out this study to elucidate whether *CP* gene variations are generally found in PD patients and contribute to the decreased levels of CP.

MATERIALS AND METHODS

Patients and Controls

Sixty PD patients were recruited from the Department of Neurology, Zhongshan Hospital, Fudan University. PD was diagnosed by two independent movement-disorder specialists (CJ Zhong and LR Jin) according to the criteria of the United Kingdom Parkinson's Disease Society Brain Bank for idiopathic PD[19] and the modified version of Hoehn and Yahr[20]. Briefly, 33% of the PD patients showed unilateral motor impairments only, corresponding to Hoehn and Yahr stage I, 47% presented bilateral or midline involvement without balance impairment (Hoehn and Yahr stage II), and 20% showed bilateral impairments with mild to moderate disability and postural reflex dysfunction (Hoehn and Yahr stage III). The PD patients were divided into three subgroups: primarily rigidity and bradykinesia with minimal tremor (PD_{AR} subgroup), primarily tremor with minimal bradykinesia and rigidity (PD_T subgroup), and mixed classic motor symptoms with propinquity proportion (PD_M subgroup) according to the ratio of average UPDRS III Tremor score of each PD patient (sum of items 20 and 21 divided by 4) to his/her average UPDRS akinetic/rigid score (sum of items 22–27 and 31 divided by 15), as previously reported[16]. Patients showing signs of upper and/or lower motor neuron impairments, orthostatic hypotension within three years since PD onset, cognitive impairment assessed by the Mini-Mental State Examination (MMSE), and hepatic and/or renal dysfunction, were excluded from this study. Fifty control participants were recruited from the Xujiahui Community of Shanghai. No controls had a history of neurologic/psychiatric disorders and cognitive impairment as assessed by the MMSE (scores ≥28).

Serum CP levels were measured in both PD patients and controls using an immunonephelometry kit (N antiserum against human CP; Dade Behring, Marburg, Germany) according to the manufacturer's instructions. Accordingly, PD patients were further divided into two subgroups: those with lower serum CP (<0.20 g/L, PD_{LCP}) and those with normal serum CP (≥0.20 g/L, PD_{NCP}).

This study was approved by the Committee on Medical Ethics of Zhongshan Hospital, Fudan University, and all participants gave informed consent.

CP Gene Sequencing

Blood samples were collected into EDTA-containing tubes. A QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from the white blood cell fraction. The sequences of all exons, intron–exon boundaries, and the DNase cluster region of the promoter in the *CP* gene were determined by aligning GenBank with genomic sequence records (NM 000096.1). The primers are shown in Table 1. *CP* amplicons were obtained by polymerase chain reaction (PCR) amplification and purified by cutting out the DNA band from agarose gel electrophoresis. DNA sequencing was performed on an ABI3730XL automated sequencer, using version 3.1 of the Big Dye fluorescent method according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequence data were analyzed using VECTOR NTI Advance 11 software (Invitrogen Corp., Carlsbad, CA).

Plasmid Construction

To construct the *CP* promoter reporter plasmid, we designed primers that could amplify the 878-bp DNA fragment containing rs66508328(GG/AA), rs67870152(CC/TT), rs16861642(GG/AA), rs73020328(TT/GG), rs11708215(GG/AA), rs73166855(AA/GG), and rs66953613(CC/TT) (Primer F: 5'-CTTGCCTGAGACCATTTTACATCC-3'; Primer R: 5'-CAACAGCACAGACTGGGGTTAG-3'). The wild-type and mutant DNA fragments were amplified from the genomic

Table 1. Primers for the *CP* gene and conditions of PCR

PCR location	Oligonucleotide of primers	Size of PCR product (bp)	Annealing temperature (°C)
Exon 1	F: 5'ACACGTTCTCTGCCCTCCTGGAA3'		
	R: 5'CGGAGATGCAGTTACGACCATGGGA3'	716	60
Exon 2	F: 5'GGAGGCATCCCTACAACAGGCA3'		
	R: 5'TGTCTTATCCAGGAGAGAAGTCCAC3'	482	62
Exon 3	F: 5'TCAGACTCCCATACCATGACCCGA3'		
	R: 5'TCACCGTGGAGTGCCCTTTTGG3'	832	63
Exon 4	F: 5'TGGTCAGTGGACATCCAGACACAGG3'		
	R: 5'ACCAGTTGGGGAACAAGTTTGGTGT3'	616	62
Exon 5	F: 5'GGCAAGAATACCAGCATGTGTGCCA3'		
	R: 5'AACAGGGTGCTTTCCAGTGCAACA3'	706	63
Exon 6	F: 5'CCAAGTGAAACCCCACAGAAACTGG3'		
	R: 5'TGCTGCTGAATCGTACAGTGCCA3'	758	63
Exon 7	F: 5' TGAGTGGACTGGAACTGTCTGCT 3'		
	R: 5' GCCCATGGGAAGAGTAAACCAGCC 3'	364	58
Exon 8	F: 5'TGACACACCTCCAGCCAACAGA3'		
	R: 5'GCGGTTTCCTTGGGAGTTCCTGC3'	592	61
Exon 9	F: 5'CCAGGAGGAGGTTTAGAAG3'		
	R: 5'GAACATTGATTGGCTATTTG3'	633	55
Exon 10	F: 5'TGTGCACATGGAAGTCTTCTGCT3'		
	R: 5'ATGAGCCTGTCATTTTTGAGCCA3'	652	60
Exon 11	F: 5'GGTCCTGGAAAGTCTGTGA3'		
	R: 5'ATCTTGAGGAGCCTATGGA3'	547	55
Exon 12	F: 5' AAAGGATGGATGGAGCAGG 3'		
	R: 5' AGCGGAAATGAATAAGGACAA 3'	532	57
Exon 13	F: 5'AGTGACTAGCTGGAGGAAAT3'		
	R: 5'AAATGAAACCCATAGACATG3'	492	59
Exon 14	F: 5'GGACTTTCAGGCCAAACCTCCCC3'		
	R: 5'ACAGACACCTCCTTGCATCCCCT3'	495	61
Exon 15	F: 5'GCTTTGTGGTATGGCAAGTGGGTT3'		
	R: 5'TCAGTGGCTACCTGTGACCCACAA3'	633	62
Exon 16	F: 5'AGCATCACCCACATGACCTACCT3'		
	R: 5'TGCTTTTCTAGGCACTTTGCACCA3'	696	60
Exon 17	F: 5'TAATCCAAAACTAAGATTAAGGC3'		
	R: 5'AATCCACGGATATGAAGCA3'	490	56
Exon 18	F: 5'GACAAACAGGCAAACCAGA3'		
	R: 5'ATCCCTCACCATTTAGCAG3'	631	55
Exon 19	1 F: 5'TCTTGTAGGGAAGAAAGTAATC3'		
	1 R: 5'GACATCTGCCACAGGTCTA3'	858	55
	2 F: 5'TGAGTTTTGTGAACCCCTGAA3'		
	2 R: 5'GGCTGCTTACCTTACCGTGTA3'	847	57
Promoter	1 F 5' AGGGAGAAGGCACTGGGAGCTAATA3'		
	1 R 5' AGGATTCAGCTAAATACTTATGCCAT3'	1260	60
	2 F 5'AGGCATAATCCCAAGTCGTTTCA3'		
	2 R 5' ATCGCATGCTTTCTTCTATACCAATC3'	420	65
	3 5' ACATGATATCTAGCATGCCAAAT3'	330	65
	4 5' AAGTTATTAGCCCCTGTTAGGCT3'	580	65

DNA of controls and patients carrying mutant single-nucleotide polymorphisms (SNPs), respectively. The DNA was constructed to the PGL3-Basic vector (Promega, San Luis Obispo, CA). All plasmids were verified by Sanger sequencing.

Cell Culture and Luciferase Assay

U251 and HepG2 cells were cultured in DMEM with 10% FBS. Then the cells were digested with trypsin and seeded in 24-well plates at 1×10^5/well with 500 μL culture medium. After 24 h in culture, 500 ng of *CP* promoter reporter plasmids were co-transfected with 10 ng pRL-TK plasmid as a normalizing control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h in culture, the cells were lysed, and 20 μL of supernatant was used to assay the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). The relative reporter activity was normalized by firefly activity to *Renilla* activity. Each assay was performed at three independent experiments with four replications.

Comparative Genomic Hybridization Microarray Analysis

We designed a high-density oligonucleotide-based comparative genomic hybridization (CGH) microarray in the Agilent 8×60K format. Briefly, we first selected 48 candidate genes associated with PD or iron/copper metabolism. Using the Agilent eArray online system, we selected 52828 oligonucleotide probes for the 48 candidates and their 150-kb flanking regions on both sides (Table S1). The genomic DNAs extracted from PD patients and sex-matched standard DNAs were fragmented *via* AluI and RsaI enzyme digestion. DNA was labeled using an Agilent SureTag DNA Labeling Kit (Agilent Technologies, Santa Clara, CA). Different fluorescent dyes were used for DNA labeling of patient DNA (Cy5-dUTP) and standard DNA (Cy3-dUTP). The labeled product from each patient was mixed with a standard product before being hybridized onto the Agilent CGH microarray (Agilent Technologies, Santa Clara, CA) for 24 h at 65°C. DNA processing, microarray handling, and scanning were conducted following the Agilent oligonucleotide CGH protocol (version 6.0). The microarray scanning profiles were processed by Agilent Feature Extraction 10.7.3.1. The extracted data were analyzed and plotted by Agilent Workbench 7.0. ADM-2 was selected as the statistical algorithm with a threshold of 6.0 and the Fuzzy Zero turned on.

Statistical Analysis

Data are presented as mean ± SEM. The unpaired *t*-test, Mann-Whitney test, or Kruskal-Wallis test (for continuous variables), or the χ^2 and Fisher exact tests (for categorical variables) were used as the main methods of statistical analysis. Genotype frequency data were also tested for Hardy-Weinberg equilibrium. Linkage disequilibrium (LD) of the *CP* gene variants was analyzed using SHEsis software (http://analysis.bio-x.cn/myAnalysis.php)[21]. Spearman correlation analysis was used to evaluate correlations. The level of significance was assumed to be 5%, and all tests were two-sided. All the statistical analyses were performed with SPSS software (version 18.0).

RESULTS

Characteristics of Study Participants

The average age was 61.52 ± 1.25 years in PD patients and 72.82 ± 0.79 years in controls. The gender ratio was 52% male and 48% female in PD patients, and 40% male and 60% female in controls. There was a statistically significant difference in the serum CP levels between PD patients (0.206 ± 0.007 g/L) and normal controls (0.224 ± 0.006 g/L) (P = 0.043). The PD patients included 28 PD_{LCP} patients (age: 60.57 ± 1.83; gender: male 71%, female 29%; serum CP level 0.165 ± 0.003 g/L) and 32 PD_{NCP} patients (age: 62.34 ± 1.73; gender: male 38%, female 62%; serum CP level 0.248 ± 0.008 g/L). The age-difference between PD_{LCP} and PD_{NCP} patients was not statistically significant (P = 0.837). There as a dramatic difference in the gender distribution in PD_{LCP} cases compared with PD_{NCP} cases (P = 0.010). Male PD patients were more prone to present low serum CP than females.

CP Gene Variants in PD Patients

The distributions and genotypes of the *CP* gene SNPs in PD patients and controls are shown in Table 2. Genotype data were first tested for Hardy-Weinberg equilibrium (HWE) before further genetic analysis. There was no significant deviation from the HWE in either the PD or control group (data not shown). Ten SNPs were found in the intronic and promoter regions of *CP*: two located at introns (rs3736282

Table 2. Distributions and genotypes of SNPs in PD cases and controls.

SNP	Location	Genotype	CTRL	PD			PD/CTRL		PD_{LCP}/CTRL	
			n=50	Total *n*=60	PD_{NCP} *n*=32	PD_{LCP} *n*=28	*P*	OR (95%CI)	*P*	OR (95%CI)
rs3736282	Intron 1	CC+CT	26+22 (96%)	29+20 (81.7%)	16+11 (84.4%)	13+9 (78.6%)	0.03	5.39 (1.13-25.61)	0.02	6.55 (1.22-35.06)
		TT	2 (4%)	11 (18.3%)	5 (15.6%)	6 (21.4%)				
rs17847023	Intron 2	GG+AG	22+25 (94%)	23+26 (81.7%)	13+15 (87.5%)	10+11 (75%)	0.08	3.52 (0.92-13.41)	0.03	5.22 (1.23-22.21)
		AA	3 (6%)	11 (18.3%)	4 (12.5%)	7 (25%)				
rs17838831	Promoter	AA+AG	21+26 (94%)	27+21 (80%)	15+11 (81.3%)	12+10 (78.6%)	0.05	3.92 (1.04-14.78)	0.06	4.27 (0.98-18.69)
		GG	3 (6%)	12 (20%)	6 (18.7%)	6 (21.4%)				
rs66508328	Promoter	GG+AG	26+23 (98%)	30+21 (85%)	17+11 (87.5%)	13+10 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		AA	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs67870152	Promoter	CC+CT	25+24 (98%)	30+21 (85%)	17+11 (87.5%)	13+10 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		TT	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs16861642	Promoter	GG+AG	25+24 (98%)	30+21 (85%)	17+11 (87.5%)	13+10 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		AA	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs73020328	Promoter	TT+TG	27+22 (98%)	32+19 (85%)	18+10 (87.5%)	14+9 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		GG	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs11708215	Promoter	GG+AG	26+23 (98%)	32+19 (85%)	18+10 (87.5%)	14+9 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		AA	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				
rs73166855	Promoter	AA+AG	22+26 (96%)	27+21 (80%)	15+11 (81.3%)	12+10 (78.6%)	0.02	3.69 (0.75-18.27)	0.02	6.55 (1.22-35.06)
		GG	2 (4%)	12 (20%)	6 (18.7%)	6 (21.4%)				
rs66953613	Promoter	CC+CT	26+23 (98%)	32+19 (85%)	18+10 (87.5%)	14+9 (82.1%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
		TT	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)				

Statistical analysis was performed using the Fisher exact test.

C>T and rs17847023 G>A) and the other eight at the promoter (rs17838831 A>G, rs66508328 G>A, rs67870152 C>T, rs16861642 G>A, rs73020328 T>G, rs11708215 G>A, rs73166855 A>G, and rs66953613 C>T). Moreover, the frequencies of eight SNPs (rs3736282 C>T, rs66508328 G>A, rs67870152 C>T, rs16861642 G>A, rs73020328 T>G, rs11708215 G>A, rs73166855 A>G and rs66953613 C>T) were significantly higher in the PD group than in the control group. Compared with controls, PD patients, especially PD_{LCP} patients, carried higher frequencies of homozygous mutant alleles of rs3736282 TT (*P* = 0.03 for PD, *P* = 0.02 for PD_{LCP}), rs66508328 AA (*P* = 0.02, *P* = 0.02), rs67870152 TT (*P* = 0.02, *P* = 0.02), rs16861642 AA (*P* = 0.02, *P* = 0.02), rs73020328 GG (*P* = 0.02, *P* = 0.02), rs11708215 AA (*P* = 0.02, *P* = 0.02), rs73166855 GG (*P* = 0.02, *P* = 0.02), and rs66953613 TT (*P* = 0.02, *P* = 0.02).

To examine the combined effect of these eight SNPs in the *CP* gene, we performed haplotype analysis (Table 3), which revealed high LD between the eight SNPs (Fig.1). The results from haplotype analysis were generally consistent with those from single SNPs; the haplotype defined by the mutant alleles of these eight SNPs (TATAGAGT) was associated with risk for PD, especially PD_{LCP}. For example, the PD patients (15%), especially PD_{LCP} (17.8%), had higher frequencies of TATAGAGT/TATAGAGT than controls (2%) (*P* = 0.02, *P* = 0.02). The distributions of the other *CP* SNP haplotypes did not differ significantly between patients and controls (Table 3).

No Association of *CP* Gene Variants with Hoehn and Yahr Stage and Motor Phenotype

We also analyzed whether the frequencies of SNPs in the

Table 3. Haplotype analysis of the block of rs3736282, rs66508328, rs67870152, rs16861642, rs73020328, rs11708215, rs73166855, and rs66953613 in PD patients and controls

Haplotype block	rs3736282	rs66508328	rs67870152	rs16861642	rs73020328	rs11708215	rs73166855	rs66953613
Haplotype	CTRL	PD			PD/CTRL		PD_{LCP}/CTRL	
	n=50	Total *n*=60	PD_{NCP} *n*=32	PD_{LCP} *n*=28	*P*	OR(95%CI)	*P*	OR (95%CI)
CGCGTGAC/CGCGTGAC 00000000/00000000	22 (44%)	25 (41.7%)	14 (43.8%)	11 (39.3%)	0.84	0.91 (0.43-1.94)	0.81	0.82 (0.32-2.11)
CGCGTGAC/TATAGAGT 00000000/11111111	20 (40%)	16 (26.7%)	9 (28.1%)	7 (25%)	0.16	0.55 (0.24-1.22)	1.00	0.95 (0.32-2.88)
TATAGAGT/TATAGAGT 11111111/11111111	1 (2%)	9 (15%)	4 (12.5%)	5 (17.9%)	0.02	8.65 (1.06-70.85)	0.02	10.65 (1.18-96.52)
TGCGTGAC/TATAGAGT 10000000/11111111	2 (4%)	2 (3.3%)	1 (3.1%)	1 (3.6%)	1.00	0.83 (0.11-6.10)	1.00	0.89 (0.08-10.27)
CGCGTGAC/CGCGTGGC 00000000/00000010	3 (6%)	2 (3.3%)	1 (3.1%)	1 (3.6%)	1.00	0.65 (0.10-4.09)	1.00	0.58 (0.06-5.86)
CGCGTGAC/CATATGAC 00000000/01110000	0 (0%)	1 (1.7%)	0 (0%)	1 (3.6%)	/	/	/	/
CGCGTGAC/TGCGTGAC 00000000/10000000	0 (0%)	2 (3.3%)	1 (3.1%)	1 (3.6%)	/	/	/	/
CGCGTGAC/TATATAAT 00000000/11110101	1 (2%)	0 (0%)	0 (0%)	0 (0%)	/	/	/	/
CGCGTGGC/CGCGTGGC 00000010/00000010	0 (0%)	1 (1.7%)	1 (3.1%)	0 (0%)	/	/	/	/
CGCGTGGC/TATAGAGT 00000010/11111111	0 (0%)	1 (1.7%)	0 (0%)	1 (3.6%)	/	/	/	/
CGCGTGGC/TATATGGC 00000010/11110010	0 (0%)	1 (1.7%)	1 (3.1%)	0 (0%)	/	/	/	/
TGCGTGAC/TGCGTGAC 10000000/10000000	1 (2%)	0 (0%)	0 (0%)	0 (0%)	/	/	/	/

0=major genotype, 1=minor genotype. Statistical analysis was performed using the Fisher exact test.

CP gene and/or haplotypes are correlated with disease severity and motor phenotypes. The results showed that the frequencies of the SNPs and haplotype did not differ significantly among the PD subgroups assayed by Hoehn and Yahr staging and by motor phenotype (Tables 4 and 5).

Promoter Activity of the *CP* Gene

Since seven of the eight significantly changed SNPs were located in the promoter region of the *CP* gene, we investigated the function of the promoter SNPs/haplotypes of the *CP* gene using the luciferase reporter system in U251 and HepG2 cells. We found no significant difference in *CP* promoter activity between the WT haplotype (GCGTGAC/GCGTGAC) and the mutant haplotype (ATAGAGT/ATAGAGT) in both U251 and HepG2 cells (Fig. 2). Although the *in vitro* luciferase reporter assay

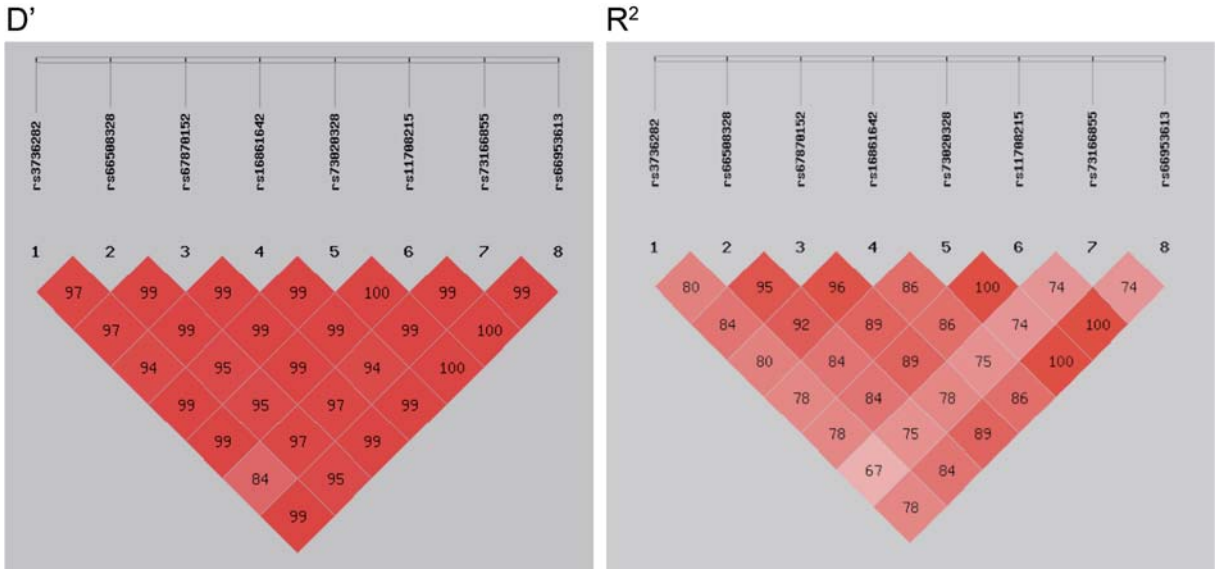

Fig. 1. Linkage disequilibrium (LD) test of *CP* gene polymorphisms (D', coefficient of LD; R^2, correlation coefficient of LD). The strength of the LD between SNPs is indicated by the color scheme. The number in each square indicates the magnitude of LD between respective pairs of SNPs. Dark red color indicating high D' and R^2; light red color indicating low D' and R^2. The blocks of dark red represent SNPs that are all in high LD with each other and thus are all inherited together.

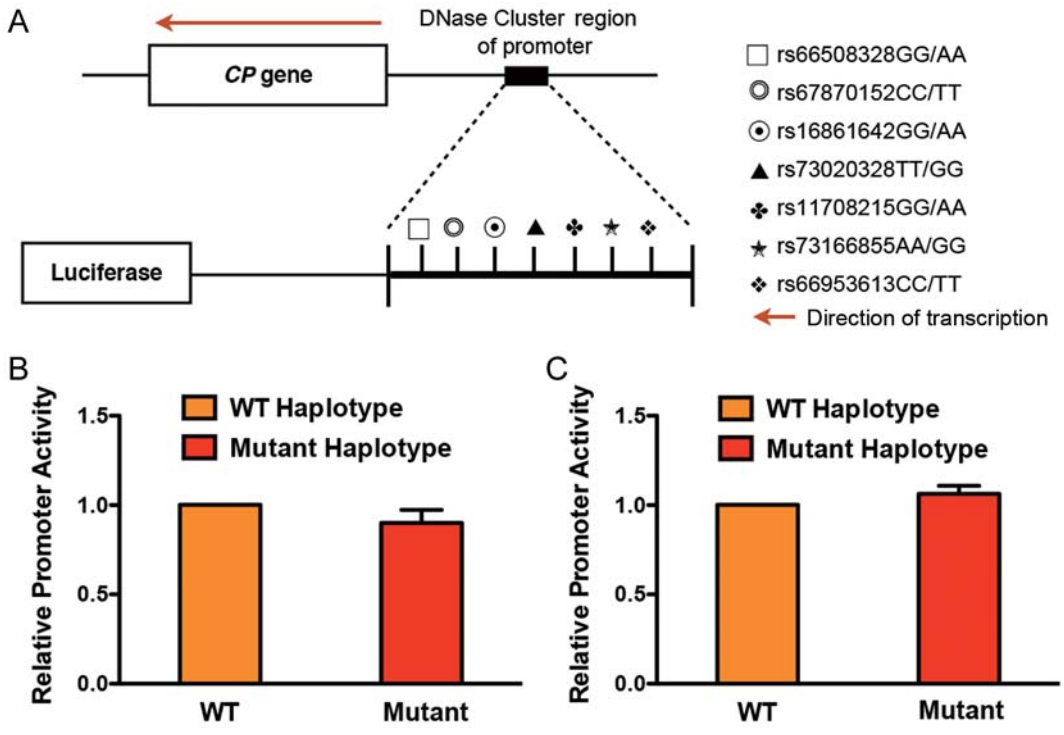

Fig. 2. Luciferase reporter assays in U251 (B) and HepG2 (C) cell lines. A. Schematic of the luciferase reporter construct with the seven SNPs represented by distinct symbols from the DNase cluster region of the *CP* gene promoter. No significant changes in *CP* promoter activity were found between the wild-type haplotype (GCGTGAC) and the mutant haplotype (ATAGAGT). Statistical analysis was performed using the unpaired *t*-test. Luciferase activity was normalized to the construct consisting of the wild-type haplotype (GCGTGAC).

Table 4. Correlation between SNPs/haplotype and PD disease severity

Subjects	Hoehn and Yahr stage I (*n*=20)	Hoehn and Yahr stage II (*n*=28)	Hoehn and Yahr stage III (*n*=12)	*P*
Age (years)	57.40±1.94	64.68±1.67	61.00±3.23	0.04
Gender	M: 11(55%)	M: 12(43%)	M: 9(75%)	
	F: 9(45%)	F: 16(57%)	F: 3(25%)	0.50
Serum CP (g/L)	0.20±0.01	0.22±0.01	0.23±0.03	0.66
Ratio of low serum CP (< 0.2g/L)	PD_{NCP}: 10(50%)	PD_{NCP}: 17(61%)	PD_{NCP}: 5(42%)	0.51
PD_{LCP}: 10(50%)	PD_{LCP}: 11(39%)	PD_{LCP}: 7(58%)		
rs3736282	CC+CT:18(90%)	CC+CT: 23(82.1%)	CC+CT: 8(66.7%)	0.26
	TT: 2(10%)	TT: 5(17.9%)	TT: 4(33.3%)	
rs17847023	GG+AG:18(60%)	GG+AG: 23(82.1%)	GG+AG: 8(66.7%)	0.26
	AA: 2(10%)	AA: 5(17.9%)	AA: 4(33.3%)	
rs17838831	AA+AG:18(90%)	AA+AG: 23(82.1%)	AA+AG: 7(58.3%)	0.09
	GG: 2(10%)	GG: 5(17.9%)	GG: 5(41.7%)	
rs66508328	GG+AG: 18(90%)	GG+AG: 25(89.3%)	GG+AG: 8(66.7%)	0.26
	AA: 2(10%)	AA: 3(10.7%)	AA: 4(33.3%)	
rs67870152	CC+CT: 18(90%)	CC+CT: 25(89.3%)	CC+CT: 8(66.7%)	0.26
	TT: 2(10%)	TT: 3(10.7%)	TT: 4(33.3%)	
rs16861642	GG+AG: 18(90%)	GG+AG: 25(89.3%)	GG+AG: 8(66.7%)	0.26
	AA: 2(10%)	AA: 3(10.7%)	AA: 4(33.3%)	
rs73020328	TT+TG:18(90%)	TT+TG: 25(89.3%)	TT+TG: 8(66.7%)	0.26
	GG: 2(10%)	GG: 3(10.7%)	GG: 4(33.3%)	
rs11708215	GG+GA: 18(90%)	GG+GA: 25(89.3%)	GG+GA: 8(66.7%)	0.26
	AA: 2(10%)	AA: 3(10.7%)	AA: 4(33.3%)	
rs73166855	AA+AG: 18(90%)	AA+AG: 23(82.1%)	AA+AG: 7(58.3%)	0.09
	GG: 2(10%)	GG: 5(11.9%)	GG: 5(41.7%)	
rs66953613	CC+CT: 18(90%)	CC+CT: 25(89.3%)	CC+CT: 8(66.7%)	0.26
	TT: 2(10%)	TT: 3(10.7%)	TT: 4(33.3%)	
TATAGAGT/TATAGAGT	2(10%)	3(10.7%)	4(33.3%)	0.14
11111111/11111111				

0=major genotype; 1=minor genotype. Statistical analysis for age, gender and the serum CP concentration was performed using the Kruskal-Wallis tests; and for the others was performed using X^2 test.

did not reveal the function of the *CP* promoter haplotype (ATAGAGT), its potential involvement in gene expression cannot be readily excluded.

No Copy Number Variations of the *CP* gene in PD Patients

Copy number variations (CNVs), including deletions and duplications, were investigated in 24 PD patients (12 PD_{LCP} and 12 PD_{NCP}) using high-density oligonucleotide CGH microarrays. Among the 48 candidate genes, we only found compound heterozygous deletions of the *PARK2* gene in one PD_{LCP} patient (Fig. 3E). No CNVs were identified in the other 47 candidate genes, including *CP*, *ATP7A*, *ATP7B*, and *ATOX1*, which are associated with iron/copper metabolism (Fig. 3A–D).

Table 5. Correlation between SNPs/haplotype and motor phenotype in PD patients

Subjects	PD_T subgroup (*n*=17)	PD_{AR} subgroup (*n*=28)	PD_M subgroup (*n*=15)	*P*
Age (years)	60.41±7.86	59.79±9.57	66.00±10.93	0.11
Gender	M: 8(47%)	M: 13(46%)	M: 11(73%)	0.20
	F: 9(53%)	F: 15(54%)	F: 4(27%)	
Serum CP (g/L)	0.23±0.08	0.21±0.06	0.20±0.05	0.27
Ratio of low serum CP (<0.2 g/L)	PD_{NCP}: 12(71%)	PD_{NCP}: 14(50%)	PD_{NCP}: 6(40%)	0.20
	PD_{LCP}: 5(29%)	PD_{LCP}: 14(50%)	PD_{LCP}: 9(60%)	
rs3736282	CC+CT: 14(82.4%)	CC+CT: 25(89.3%)	CC+CT: 12(80%)	0.67
	TT: 3(17.6%)	TT: 3(10.7%)	TT: 3(20%)	
rs17847023	GG+AG: 13(76.5%)	GG+AG: 24(85.7%)	GG+AG: 11(73.3%)	0.60
	AA: 4(13.5%)	AA: 4(14.3%)	AA: 4(26.7%)	
rs17838831	AA+AG: 11(64.7%)	AA+AG: 23(82.1%)	AA+AG: 11(73.3%)	0.42
	GG: 6(35.3%)	GG: 5(17.9%)	GG: 4(26.7%)	
rs66508328	GG+AG: 14(82.4%)	GG+AG: 25(89.3%)	GG+AG: 12(80%)	0.78
	AA: 3(17.6%)	AA: 3(10.7%)	AA: 3(20%)	
rs67870152	CC+CT: 14(82.4%)	CC+CT: 25(89.3%)	CC+CT: 12(80%)	0.78
	TT: 3(17.6%)	TT: 3(10.7%)	TT: 3(20%)	
rs16861642	GG+AG: 14(82.4%)	GG+AG:25(89.3%)	GG+AG: 12(80%)	0.78
	AA: 3(17.6%)	AA:3(10.7%)	AA: 3(20%)	
rs73020328	TT+TG: 14(82.4%)	TT+TG: 25(89.3%)	TT+TG: 11(73.3%)	0.41
	GG: 3(17.6%)	GG: 3(10.7%)	GG: 4(26.7%)	
rs11708215	GG+GA: 14(82.4%)	GG+GA: 25(89.3%)	GG+GA: 11(73.3%)	0.41
	AA: 3(17.6%)	AA: 3(10.7%)	AA: 4(26.7%)	
rs73166855	AA+AG: 14(82.4%)	AA+AG: 23(82.1%)	AA+AG: 11(73.3%)	0.97
	GG: 3(17.6%)	GG: 5(17.9%)	GG: 4(26.7%)	
rs66953613	CC+CT: 14(82.4%)	CC+CT: 25(89.3%)	CC+CT: 11(73.3%)	0.41
	TT: 3(17.6%)	TT: 3(10.7%)	TT: 4(26.7%)	
TATAGAGT/TATAGAGT	3(17.6%)	4(14.3%)	2(13.3%)	0.98
11111111/11111111				

0=major genotype; 1=minor genotype. Statistical analysis for age, gender and the serum CP concentration was performed using the Kruskal-Wallis tests; others were performed using X^2 test.

DISCUSSION

CP gene mutation of a 5-bp insertion at amino-acid 410 in exon 7 resulting in a frame-shift mutation and a truncated open reading frame was reported by Harris *et al.* in 1995[5]. The patient presented with parkinsonism and had a total absence of circulating serum CP with retinal and basal ganglia iron deposition caused by the genetic defect of the *CP* gene. Another nonsense mutation of this gene (Trp858ter) was reported by Miyajima in a patient who presented with cerebellar ataxia and hypoceruloplasminemia, as well as iron deposition in the basal ganglia[22]. The presence of the mutation in conjunction with the clinical and pathologic findings

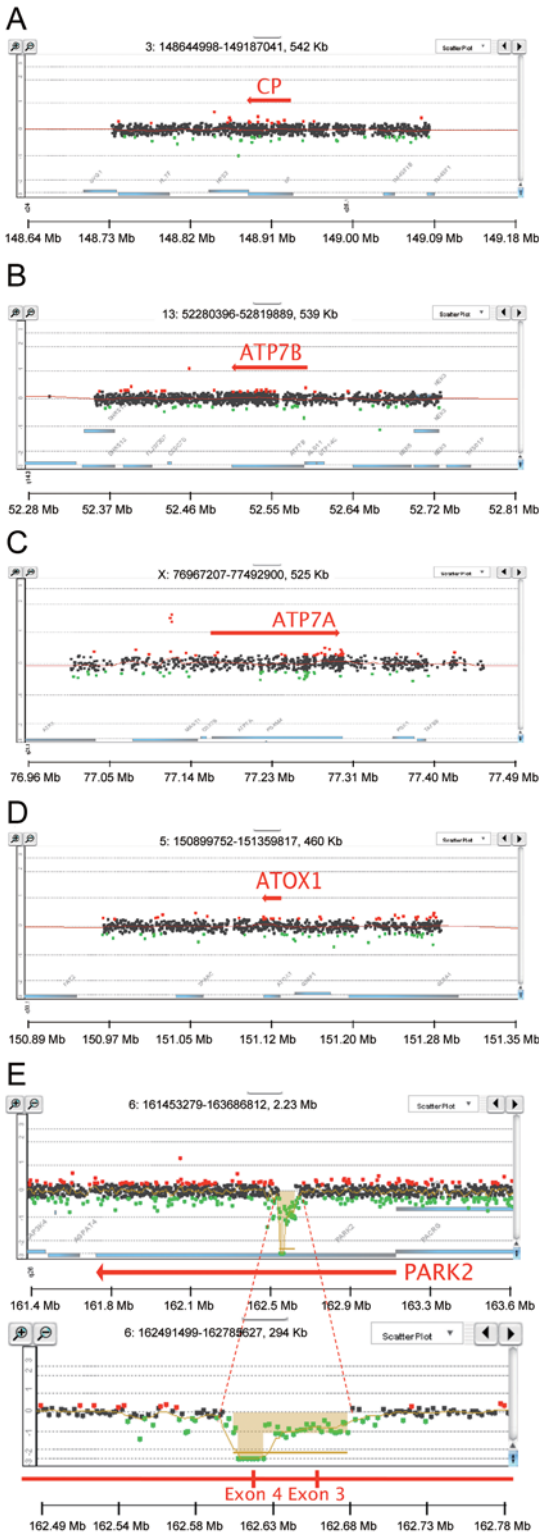

Fig. 3. CNV analysis. The CGH microarray results are shown for the *CP*, *ATP7A*, *ATP7B*, *ATOX1* and *PARK2* genes and their flanking regions.

demonstrated an essential role of CP in iron metabolism in the central nervous system. The fact that Cp-knockout mice develop parkinsonism and are rescued by iron chelation further demonstrates the role of CP in the pathogenesis of PD[12].

Previous studies from our group and others also demonstrated that decreased serum CP levels are associated with movement disorders, including PD, by disturbing brain iron metabolism[14, 15, 17, 23]. However, less is known about the cause of low serum CP levels in PD patients. In this study, we found that the *CP* gene in controls and PD patients had a number of SNPs located at the introns and promoter region, but no exonic SNPs were found. The frequencies of eight SNPs of the *CP* gene (rs3736282 C>T, rs66508328 G>A, rs67870152 C>T, rs16861642 G>A, rs73020328 T>G, rs11708215 G>A, rs73166855 A>G and rs66953613 C>T) and their haplotype (TATAGAGT/TATAGAGT) defined by the mutant alleles of these SNPs in PD patients, especially PD_{LCP} patients, were significantly higher than those in controls, implying a possible genetic risk for PD.

Hochstrasser and colleagues screened the entire coding region of the *CP* gene in 176 German patients with idiopathic PD and 180 ethnically-matched heathy individuals, and found six missense variants in the coding region: I63T, P477L, D544E, T551I, R793H, and T841R. The frequency of D544E differed significantly between PD patients and controls[24]. Another study by Castiglioni *et al.* of 103 Italian PD patients revealed 24 nucleotide substitutions, of which 11 were in the coding region, one in the 3′ UTR, and 12 in the introns. The D544E substitution, which was previously found to be associated with PD, was not significantly different from that reported in dbSNP of Pubmed and similar to the control population of 180 individuals reported by Hochstrasser. None of the other 23 *CP* gene variants seemed to be associated with PD in this study[25]. In another study involving 21 Mexican PD patients and 13 healthy volunteers, Martınez-Hernandez *et al.* found no D544E mutation of the *CP* gene in the PD patients[17]. Thus, the *CP* gene variants in PD patients are inconsistent in different reports. Compared with these studies, we did not find any exonic variants of the *CP* gene like those in Martınez-Hernandez's report, but two intronic SNPs (rs3736282 and rs17847023) consistent with Castiglioni's report[25], as well as eight promoter SNPs. These differences may be explained by the different ethnicity and

location of the participants. Another important factor is the problem of our small sample size.

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Thus, we further explored the promoter activity of the *CP* gene using a dual luciferase reporter system transfected with the wild-type haplotype and mutant haplotype DNA fragments in U251 and HepG2 cells which have abundant *CP* gene expression, in order to verify the function of *CP* gene variants, especially the promoter haplotype. No significant ELECTRONIC difference was found in the promoter activity between the wild-type and mutant haplotype in both U251 and HepG2 cells. However, this luciferase reporter system was affected by the specificity of cells, thus it might not precisely reflect the fact of gene regulation *in vivo*. Thus, further investigation is needed to explore the association of *CP* gene variants and CP levels in PD.

Human genomic rearrangement can cause CNVs[18]. Rare CNVs are important genetic causes of human diseases, especially neurological disorders including PD[26-32]. Therefore, defining the genetic content and genomic location by high-resolution CNV breakpoint analysis is vital to elucidating the etiology of CNV-associated disorders. The *PARK2* gene is a molecular diagnostic test for parkinsonism. Kay *et al.* reported that a total of 0.95% of controls and 0.86% of patients carry a heterozygous CNV mutation of the *PARK2* gene[30]. Thus, there is no compelling evidence for an association of heterozygous *PARK2* gene CNV mutation with PD. The compound heterozygous deletions of the *PARK2* gene found in one PD_{LCP} patient in our study did not indicate a relationship with the low CP level in PD. Although no CNVs of the *CP* gene were identified in this study and others, their potential involvement in low serum CP and/or PD cannot be readily excluded. Further studies in more human populations will be informative for revealing more genetic risk factors of PD.

In conclusion, this study demonstrated CP genetic variants associated with PD, especially in PD_{LCP} patients. To our knowledge, this is the first study to reveal such variants and their relation with serum CP levels in a Chinese population. More in-depth studies in larger populations from this and other ethnic groups are needed. In addition, further investigations of epigenetic changes, like aberrant methylation of cytosine residues in genomic DNA, are needed to explore their contribution to PD and the decreased CP expression in PD patients. Furthermore, microRNAs that act as post-transcriptional regulators of gene expression have been recognized as contributors to pathological states in PD[33-35]. For instance, miR-133b is involved in PD through regulating the expression of paired-like homeodomain transcription factor 3 (Pitx3)[36]. Further investigation of the expression of microRNAs that might regulate CP gene expression in PD patients is needed.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s12264-014-1512-6.

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·Review·

Novel drug-delivery approaches to the blood-brain barrier

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The blood-brain barrier (BBB) maintains homeostasis by blocking toxic molecules from the circulation, but drugs are blocked at the same time. When the dose is increased to enhance the drug concentration in the central nervous system, there are side-effects on peripheral organs. In recent years, genetic therapeutic agents and small molecules have been used in various strategies to penetrate the BBB while minimizing the damage to systemic organs. In this review, we describe several representative methods to circumvent or cross the BBB, including chemical and physical strategies.

Keywords: drug delivery; blood-brain barrier; nanoparticles; focused ultrasound

Introduction

Treatment of central nervous system (CNS) diseases is challenged by the difficulty in drug delivery through the blood-brain barrier (BBB). Although drug mechanism research has become quite sophisticated in recent years, the vast majority of drugs are blocked by the BBB and thus fail to reach the brain, making it difficult to treat intracranial disease[1].

The BBB has the function of selective permeability which prevents bacteria and other pathogenic microorganisms from entering the brain while at the same time allowing oxygen and other vital compounds to traverse from the blood to the brain. However, it also fends off drugs, and so is an obstacle to treating brain diseases.

Methods for drug delivery to the brain can be divided into two types: invasive and non-invasive. The invasive methods can achieve a high local drug concentrations by direct injection or intracerebroventricular delivery but also has side-effects such as infection or trauma. Besides, the drug concentration decreases exponentially as a function of distance from the injection site. It is also hard to deliver drugs repeatedly and patients hesitate to accept invasive treatments. For these reasons, we focus on the non-invasive drug-delivery strategies.

Structure of BBB

The BBB is a layer of endothelial cells on the basement membrane lining almost 99% of the brain capillary surface, continuously coupled with perivascular cells[2], such as pericytes, smooth muscle cells, astrocytes, and microglia (Fig. 1)[3]. Normally the BBB excludes ionic water-soluble drugs with a diameter >180 nm[4].

Transplantation studies have shown that the properties of the endothelial cells that constitute the BBB are not innate[5], but are induced in the special microenvironment of the CNS[6]. The BBB is formed during embryogenesis when endothelial cells enter the CNS. One week before astrocyte formation, pericytes are recruited to the neonatal vessels and regulate the functions of the BBB, including the generation of tight junctions (TJs) and vesicle trafficking in brain microvascular endothelial cells (BMECs)[7-9], a major component of the BBB[10]. Pericytes are a prerequisite

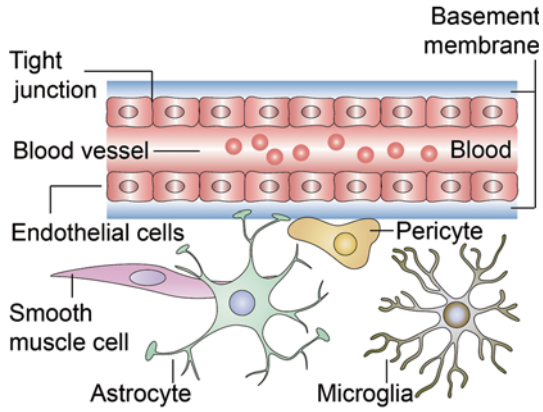

Fig. 1. Diagram of BBB. The BBB is a layer of endothelial cells on the basement membrane lining almost 99% of the brain capillary surface, continuously coupled with perivascular cells, such as pericytes, smooth muscle cells, astrocytes, and microglia.

for the formation of the BBB and determine part of its permeability by inhibiting the expression of molecules that increase BBB permeability and immune cell infiltration. However, they do not induce BBB-specific gene expression in CNS endothelial cells[11, 12]. Astrocytes induce BBB formation after birth because of the close spatial relation between astrocytes and BMECs. The timing of BBB formation has been controversial. Laboratory mice with null and hypomorphic Pdgfrb alleles that have defects in pericyte generation illustrated that the interactions between pericytes and BMECs are critical in regulating BBB permeability. This effect is caused by the inhibition of specific proteins that can increase the permeability of the BBB[13-15].

The permeability of the BBB in drug delivery remains a problem, although many drugs have been developed in an attempt to combat it. Several available strategies for the safe and effective delivery of drugs are described below.

A Drug-Delivery Approach to Bypassing the BBB

Intranasal delivery of drugs is a potential strategy to bypass the BBB[16]. The effectiveness of intranasal delivery is determined by administration factors and physicochemical properties, such as the patient's head position, dosing device, drug volume, pH value, osmotic pressure, and drug solubility. Intranasal delivery has been highly regarded because it is noninvasive, safe, and simple. Since its early use by William Ewart[17] for the treatment of diphtheria, intranasal delivery has been confirmed as a promising route of administration. On the other hand, its use is relatively limited. However, the method has been modified by various additions such as penetration enhancers, adhesion agents, and nanoparticles, which can significantly increase the efficiency of drug delivery. Wu *et al*.[18] have successfully delivered stem cells using the intranasal approach as a therapy for experimental allergic encephalomyelitis in rats, an animal model of multiple sclerosis. Nasal glucagon-like peptide-1[19] has already been used in patients. This is a promising development for patients with diabetes, and has the potential that insulin may be administered in a similar way. Future research is needed to further reveal the mechanisms of nasal drug delivery and at the same time improve the technology and solution preparation. This will achieve a better targeting, improved effectiveness, and higher drug concentrations.

New Drug-Delivery Approaches to Crossing the BBB

Relevant Carriers in Cerebral Microvascular Endothelia

Receptors on the surface membranes of cells can help drug delivery. Common carriers include medium-chain fatty-acid carriers, neutral amino-acid carriers, a monocarboxylic carrier, cation transporters, and the adenosine purine carrier.

Exosomes[20] Scientists at the University of Oxford have used protein carriers called exosomes to transport drug molecules to the brain cells of laboratory mice. Exosomes are membrane vesicles released by a variety of cells such as dendritic cells[21,22]. They transport material back and forth through the BBB. Exosomes are first extracted from mice. Then, a CNS-specific rabies viral glycoprotein is attached to the acetylcholine receptor, and fused to the exosomes. Finally, an siRNA is placed in the exosomes and the complex is intravenously injected into mice. Experiments have confirmed that the siRNA is delivered to the brain and binds to its receptors on brain cells. This results in a 60% decline of β-secretase 1 (BACE1) expression, a gene associated with Alzheimer's disease[23].

Adenosine receptor Researchers at Cornell University found that adenine nucleotide can transport large molecules into the brain. When the adenine nucleoside receptors on cells are activated, a channel can be established through the BBB[24]. In the experiments, the team succeeded in passing large molecules (a β-amyloid protein antibody) through the BBB of transgenic mice and reported the adhesion of the antibody to β-amyloid plaques (mice with genetically-modified plaques that have a lower risk of Alzheimer's disease). Furthermore, the selective A2A adenosine receptor agonist Lexiscan can temporarily open BBB channels.

Transferrin receptor[25] The transferrin receptor (TfR) is a key cell-surface molecule that regulates the uptake of iron-bound transferrin[26]. Plasma soluble TfR concentrations reflect the receptor density on cells and the number of cells expressing the receptor. Therefore, it is closely related to cellular iron demand and the erythroid proliferation rate. TfR is frequently overexpressed in cancer cells[27]. Recently, transferrin-targeted conjugates have shown promise in reversing drug resistance in cancer cells, and transferrin immunotoxins with a diphtheria toxin mutant covalently bound to transferrin have shown promise for the treatment of glioblastoma in clinical trials[28]. Thus, intracellular targeting by iron-saturated transferrin as a ligand for TfR-mediated endocytosis has become a focus of research. The natural receptor TfR has been used by Roche; therapeutic antibodies are attached to TfRs in a modified pattern, which they call a “Brain Shuttle Module”. Monovalent binding to the TfR instead of bivalent binding, which causes lysosome sorting, can lead to a reduction of amyloid. This is a process of “receptor-mediated transcytosis”.

Nanoparticles[29]

Nanoparticles make up solid colloids composed of polymers or lipid particles of 10–1000 nm (usually 50–300 nm). A drug can be embedded within a particle’s substrate or attached to its surface[30]. Drugs are transported in a controlled time period to a targeted location *in vivo*. During this process, certain principles should be followed: nanoparticles used as drug carriers should be non-toxic, biodegradable, and biocompatible; have a diameter <100 nm and no aggregation reaction in blood, as well as an efficient production process[31].

Poly-nanoparticles such as PBCA-NPs[32-35] (butylcyanoacrylate), PEG[36-40] (polyethylene glycol), liposomes[41-43], P-gp (P-glycoprotein), and even superparamagnetic iron oxide nanoparticles[37] have been used for drug delivery.

To investigate the mechanisms behind nanoparticles, it must be recognized that materials on the nanoscale take on new biological and physical characteristics. For example, there may be a ubiquitous toxic effect on BMECs. A surfactant effect due to the solubilization of lipids in the endothelial cell membrane may lead to membrane fluidization and therefore enhanced drug permeability of the BBB.

Opening TJs between BMECs can allow drugs to pass through the BBB alone or with nanoparticles. Another option is receptor-mediated endocytosis followed by transcytosis into the CNS or drug release in endothelial cells[31].

Adjustment of Tight Junctions[44] between Endothelial Cells of the BBB

There are three means of barrier disruption (Table 1): osmotic, pharmacological, and mechanical (focused ultrasound (FUS) with microbubbles).

Many drugs are slow to exert an effect, as has been shown in *in vitro* studies[51]. This is due to the low drug concentration caused by the BBB. The most direct way to increase drug permeability of the BBB is to open the TJs between endothelial cells. FUS can temporarily open the BBB and its efficiency is optimized when combined with microbubbles. FUS-induced BBB disruption occurs with sonication most of the time[52]. Drug delivery by this method has been verified by extensive research[53-57].

The mechanisms by which ultrasound opens the BBB rely on various physical characteristics and are closely associated with biological processes. Electron microscopy has confirmed that ultrasound causes enlarged biomembrane lacunae with no evident tissue damage both *in vivo* and *in vitro*. It has also been confirmed that after ultrasound irradiation, the capillary permeability increases, including endocytosis, opening of TJs, and free transportation through the endothelial lacunae[58].

FUS is often used in oncotherapy, but it has not reached the same level of maturity in the field of BBB-opening. FUS is noninvasive and precise, causes only local damage, is time-efficient, and is secure and repeatable in operation. A high dose of ultrasound has mild direct

Table 1. Details of methods of barrier disruption

	Osmotic	Pharmacological	Mechanical
Device	/	/	Focused ultrasound
Appearance	1970s[45]	1980s[46]	1940s for noninvasive ablation in brain[47]
Reagent	Hypertonic solution of 25% mannitol	Bradykinin[48] RMP-7[49]	Nano-microbubbles
Principal	Shrink endothelial cells and disrupt tight junctions between them	Bind to receptors, temporarily increase Ca^{2+} inflow, activate nitrogen oxidase, cytoskeletal contraction	Physical effects of ultrasound
Advantages	Effective in experimental and clinical applications	Used with antineoplastic drugs to amplify drug efficiency	Noninvasive; volume of drug, extent and degree of barrier disruption can be pre-established by parameter setting[50]; drug-loaded microbubbles for better targeting.
Disadvantages	Risk of high-speed delivery into arterial circulation of brain	Mainly for brain-tumor barrier	Requires more trials on parameter setting.

cytotoxic effects. Injuries mostly occur in blood vessels and epithelial cells resulting in a targeted zone of oxygen deficiency[59]. Research is focused on adjusting the parameters of FUS to make it effective in drug delivery[60].

Tunneling Nanotubes

The tunneling nanotube (TNT)[61-65] is a new general communication method between mammal cells. TNTs are somewhat similar to the protoplasmic connections in plants, but they differ in structure and function. TNTs have already been used to transport particles outside or inside the BBB[66]. In particular, mitochondria are the most common particles transported from one cell to another through TNT (Fig. 2)[67-71].

Interestingly, researchers at UCLA's Jonsson Comprehensive Cancer Center found that RNA can be transported into mitochondria, but little is known about the mechanism. They found that polynucleotide phosphorylase (PNPASE) protein[72, 73] plays an important role in transporting RNA into mitochondria. When the expression of PNPASE is reduced, the amount of RNA entering mitochondria declines; PNPASE affects the RNA-encoding process of the mitochondrial genome and the synthesis of proteins necessary to sustain electron transfer. When PNPASE expression is reduced, mitochondrial

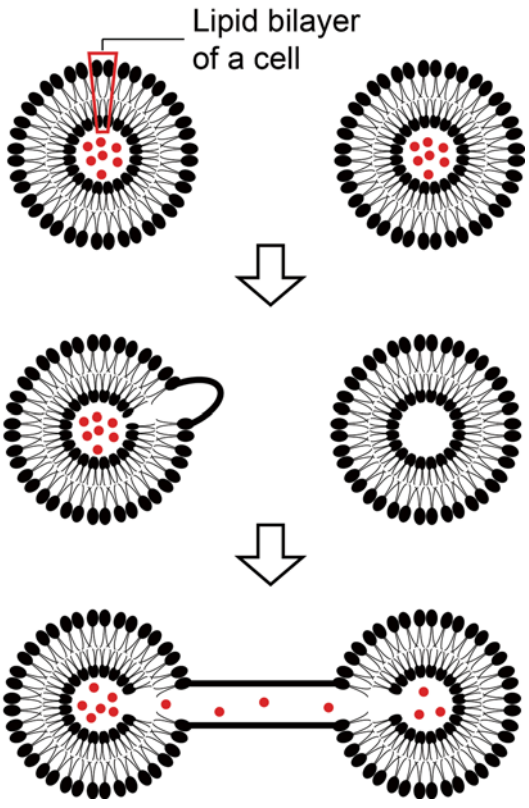

Fig. 2. Diagram of formation of tunneling nanotube between two mammal cells. Red dots: mitochondria.

RNA accumulates, unprocessed protein translation is suppressed, and energy generation is hampered, leading to the arrest or inhibition of cell growth. According to the research, PNPASE mediates the transport of cytoplasmic RNA for energy production by mitochondria. However, no experiment using current detection methods has been able to confirm this theory. If we could combine TNTs with PNPASE-dependent RNA, import them into mitochondria, and transport mitochondria into BMECs[74] transcending the TJs between them, a direct route to brain would be available.

Conclusion

The BBB impedes the entry of many drugs into the CNS. Although these drugs are somewhat effective, they have not been used in clinical treatments due to the low solubility, chemical instability, low bioavailability, and harmful side-effects. These limitations restrict their clinical applications, leaving many CNS diseases poorly treated. Over the past 20 years, many experiments have been conducted to solve these problems. One main goal is to discover a way to deliver drugs across the BBB safely, effectively, and noninvasively.

Defects still exist in every drug-delivery strategy. For example, in nasal delivery, drug molecules can only stay in the nasal cavity for 15–20 min due to ciliary clearance, and are often not fully absorbed before clearance. In addition, nasal delivery may increase the circulating concentration through absorption by the respiratory or olfactory mucosa, causing decreased efficiency in brain targeting. Although the experiments described above are still in the initial stages and the data need to be verified, they inspire various possibilities for breakthroughs in the field of BBB permeability.

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·Method·

Preserving GABAergic interneurons in acute brain slices of mice using the N-methyl-*D*-glucamine-based artificial cerebrospinal fluid method

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ABSTRACT

Defects in the function and development of GABAergic interneurons have been linked to psychiatric disorders, so preservation of these interneurons in brain slices is important for successful electrophysiological recording in various *ex vivo* methods. However, it is difficult to maintain the activity and morphology of neurons in slices from mice of >30 days old. Here we evaluated the N-methyl-*D*-glucamine (NMDG)-based artificial cerebrospinal fluid (aCSF) method for the preservation of interneurons in slices from mice of up to ~6 months old and discussed the steps that may affect their quality during slicing. We found that the NMDG-aCSF method rescued more cells than sucrose-aCSF and successfully preserved different types of interneurons including parvalbumin- and somatostatin-positive interneurons. In addition, both the chemical and electrical synaptic signaling of interneurons were maintained. These results demonstrate that the NMDG-aCSF method is suitable for the preservation of interneurons, especially in studies of gap junctions.

Keywords: artificial cerebrospinal fluid; acute brain slice; electrophysiology; N-methyl-*D*-glucamine; parvalbumin; somatostatin

INTRODUCTION

GABAergic interneurons inhibit neuronal depolarization by releasing γ-aminobutyric acid (GABA). Dysfunction of these interneurons may be responsible for some psychiatric/neurological disorders, such as schizophrenia, autism, and epilepsy, so their electrophysiological properties have been intensively studied. One of the major research methods is patch-clamp recording in brain slices *in vitro*. The efficiency of this method mainly relies on the quality of the slices. To obtain good-quality slices, Na^+ in the artificial cerebrospinal fluid (aCSF) was initially replaced with equimolar sucrose[1]. Later, other Na^+ substitutes were introduced, including N-methyl-*D*-glucamine (NMDG)[2-4], glycerol[5], Tris, and choline. However, apart from the NMDG and choline substitutes, they are only adequate for mice younger than ~30 days. Choline substitution in aCSF results in the morphological preservation of slices from mice >3 months old and up to years of age, but the electrophysiological properties of neurons in these slices are lost. In contrast, the NMDG-based aCSF method not only enables morphological preservation of brain slices of mice over a lifespan similar to the choline-based method, but also preserves the function of chemical synapses[3,4]. The NMDG-aCSF method was initially described for studying glial cells in the spinal cord[2]. Later, it was introduced for the preparation of brain slices. Recently, additional supplements such as N-acetyl-L-cysteine (NAC) and glutathione ethyl-ester have been incorporated into the NMDG-aCSF method, resulting in the enhanced maintenance of slices *in vitro* for up to 12 h[3,4,6].

To facilitate studies of GABAergic interneurons, in this study we attempted to preserve their cellular morphology and electrophysiological properties using the NMDG-

aCSF method. We evaluated the reproducibility of this method, focusing on its effect on parvalbumin (PV)- and somatostatin (SST)-positive interneurons, which account for ~70% of all interneurons.

MATERIALS AND METHODS

Ethical Statement

All animal experiments were approved and reviewed by the Animal Advisory Committee at Zhejiang University in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Mice

The following mice were used throughout the experiments: GIN mice (#003718, The Jackson Laboratory, Bar Harbor, ME) expressing enhanced green fluorescent protein (eGFP) in a group of SST interneurons under the control of the *Gad1* promoter, and G42 mice (#007677, The Jackson Laboratory) expressing eGFP in a subclass of PV-positive interneurons under the control of the *Gad1* promoter. A CCK-Cre-ER^{T2} (#012710, The Jackson Laboratory) mouse was crossed with an Ai9 mouse (#007909, The Jackson Laboratory) to produce mice expressing the fluorescent protein tdTomato under the control of the *Cck* promoter in cortical neurons (both interneurons and pyramidal neurons). These offspring are referred to as "CCK-TOM".

Preparation of Acute Brain Slices

Generally, for the sucrose aCSF method, mice were anesthetized by ether inhalation and then were perfused with ice-cold sucrose-aCSF containing (in mmol/L): 185 sucrose, 2.5 KCl, 25 *D*-glucose, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, and 10 $MgCl_2$, pH 7.35 by NaOH or HCl. The brain was cut with a microtome (Leica VT1200S with vibrocheck, Nussloch, Germany) and slices (300 μm) were maintained in oxygenated standard recording aCSF at 34°C for 20 min and subsequently kept in standard recording aCSF at 22°C for at least 30 min until the experiment. The standard recording aCSF contained (in mmol/L): 124 NaCl, 2.5 KCl, 13 *D*-glucose, 24 $NaHCO_3$, 1.2 NaH_2PO_4, 5 HEPES, 2 $CaCl_2$, and 2 $MgSO_4$, pH 7.35 by NaOH or HCl.

For the NMDG aCSF method, acute slices were prepared based on the description by Guoping Feng at MIT[3,4]. Briefly, mice were deeply anesthetized by ether inhalation and then cardiovascular perfusion was performed using ice-cold NMDG aCSF consisting of (in mmol/L): 93 NMDG, 93 HCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 25 *D*-glucose, 20 HEPES, 5 Na-ascorbate, 2 thiourea, 3 Na-pyruvate, 10 $MgSO_4$, and 0.5 $CaCl_2$, pH 7.35 with NMDG or HCl. The brain was removed and placed into NMDG aCSF (0–2°C) bubbled with 95% O_2/5% CO_2. Slices were cut followed by primary recovery in oxygenated NMDG aCSF at 34°C for 10–12 min and secondary recovery in oxygenated HEPES aCSF at 22°C for >60 min. The HEPES aCSF contained (in mmol/L): 92 NaCl, 30 $NaHCO_3$, 25 *D*-glucose, 2.5 KCl, 1.2 NaH_2PO_4, 20 HEPES, 5 Na-ascorbate, 2 thiourea, 3 Na-pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$, and 12 NAC, pH 7.35 by NaOH or HCl. Slices from the prefrontal cortex, hippocampus, and midbrain were used for electrophysiological and imaging experiments. The osmolality of each aCSF was ~310 mOsm.

Electrophysiology

Individual slices were maintained in a recording chamber perfused with oxygenated standard recording aCSF (1–2 mL/min) throughout the experiment. Whole-cell paired patch-clamp recording was performed using an intracellular solution containing (in mmol/L): 110 K-gluconate, 40 KCl, 10 HEPES, 3 Mg-ATP, 0.5 Na_2-GTP, and 0.2 EGTA, pH 7.25 with KOH or HCl. Signals were acquired using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) with a Digidata 1440A (Molecular Devices) controlled by Clampex 10.4. Signals were filtered at 2 kHz for voltage clamp and 3 kHz for current clamp, and digitized at 10 kHz. Electrodes were made from borosilicate glass (with filament, Sutter, Novato, CA) with a resistance of 3.5–4.5 MΩ. Whole-cell patch-clamp was formed after a gigaohm seal was achieved with series resistance <20 MΩ. Chemical synaptic events were evoked by alternate field stimulation (square-wave pulses of 100 mV, 1000 Hz, duration 1 ms, the stimulating electrode was >50 μm apart from the recording pipette) with a pencil-shaped concentric bipolar electrode (outer pole 125 μm, inner pole 25 μm, rounded tip, #CBARC75, FHC, Bowdoin, ME). With the same pipette solution as above, both the inhibitory and excitatory postsynaptic currents were inward. Cells were held at −70 mV to detect unitary postsynaptic currents or miniature postsynaptic currents. For electrical coupling, we simultaneously patched two eGFP-positive cells (<20 μm apart). Cells were held under the current-clamp mode and resting membrane potential was maintained at −70 mV

with continuous current injection. Electrical coupling was evoked by 500 ms of hyperpolarizing current injection (−100 pA) into each cell alternately. The threshold for electrical coupling confirmation was 1% (ratio of the steady-state voltage deflection of coupling cell ΔV_2 and injected cell ΔV_1).

Imaging

During the electrophysiology experiments, neurons were identified with either a Zeiss Achroplan 10×/0.25 Ph1 lens or a Zeiss Achroplan IR 40×/0.80 W lens mounted on an upright Zeiss Axioskop 2 FS mot platform with a mercury lamp. Images were captured with either a DAGE-MTI IR-1000 Monochrome camera or a Zeiss LSM 5 Exciter system.

RESULTS AND DISCUSSION

To evaluate and compare the NMDG-aCSF method with other methods, we first examined morphological preservation. In NMDG-aCSF, cells in the prefrontal cortex, hippocampus, and dorsal raphe nucleus of the midbrain displayed clear and plump cell bodies while in sucrose-aCSF the cells swelled and died (Fig. 1A). In addition, confocal images clearly showed the morphology of different types of neurons in CCK-TOM, G42, and GIN mice (Fig. 1B and C). The improved morphological preservation using the NMDG aCSF method was most likely due to relief from oxidative stress. Such stress can induce edema[7-9] and activate glutamate receptors resulting in acidosis[10]. These events lead to irreversible membrane damage such as lipid peroxidation, while replenishing the endogenous antioxidants can relieve these processes. Regular antioxidants include sodium pyruvate[11], sodium ascorbate[7], thiourea[12] and HEPES[13]. However, in aging mice the effect is not strong enough to preserve the health of slices, so the very powerful endogenous antioxidant, glutathione, was introduced[6,14]. This antioxidant rescues neurons from degeneration while simultaneously maintaining synaptic plasticity[15]. As glutathione is not membrane-permeable, NAC is introduced. NAC is membrane-permeable and is converted into glutathione

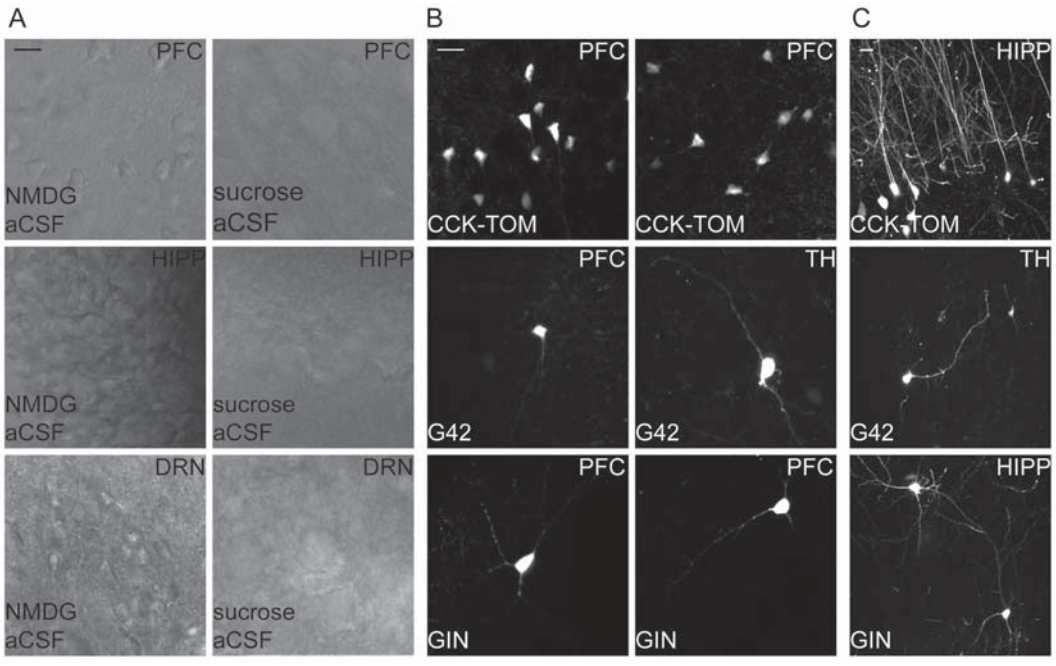

Fig. 1. Morphological preservation of neurons in brain slices. (A) Direct comparison of neurons (2–3 months old) from the prefrontal cortex (PFC), hippocampus (HIPP) and dorsal raphe nucleus (DRN) using the NMDG and sucrose aCSF methods. More neurons swell and die when prepared with sucrose aCSF. (B, C) Confocal microscopic images of neurons from the PFC, hippocampus, and thalamus (TH) from 3 transgenic mice (3–6 months old). Neurons display clear, healthy, and branched projections. Scale bars, 20 μm.

in the cytosol, providing on-line *de novo* glutathione synthesis[3,4,14]. As indicated by Jonathan Ting at the lab of Guoping Feng, NAC treatment is able to preserve the morphology and functionality of neurons for up to 12 h (www.brainslicemethods.com)[3,4]. We also found that NAC stabilized cellular health and prolonged survival time. Although it has been suggested that NAC should be applied throughout the slice preparation process, limiting NAC to the second HEPES aCSF recovery step was sufficient to keep slices alive and healthy for at least ~6 h. Once slices were transferred to the recording chamber, they could stay alive for another 3–4 h. Another more effective but expensive alternative to NAC is glutathione ethyl-ester, which is a membrane-permeable glutathione that is able to directly replenish endogenous glutathione depletion. The explanation for the strong beneficial effects of NMDG on slices is not directly evident. Based on previous reports, NMDG is less membrane-permeable than Na^+ and K^+[16]. Thus, replacement of either Na^+ or K^+ with extracellular NMDG hyperpolarizes the resting membrane potential[17]. The hyperpolarized resting membrane potential can keep neurons in a more “silent” mode, which might explain the protective effect of NDMG aCSF.

Besides morphological preservation of neurons in slices with the NMDG aCSF method, we also investigated the preservation of electrophysiological properties. Neurons of different types (Fig. 2A) and at different locations (Fig. 2B) were able to generate regular firing patterns. In contrast to the choline aCSF preservation method, in which electrical activity is lost, neurons prepared with the NMDG aCSF method displayed normal Na^+, Ca^{2+}, and K^+ currents (Fig. 2C). Moreover, we applied field stimulation to the Schaffer collaterals and detected postsynaptic currents in the CA1 region (Fig. 2D upper panel). Together with the miniature postsynaptic current recordings (Fig. 2D lower panel), these results demonstrated the presence of chemical coupling. Besides their chemical connections, we determined whether gap junctions were also preserved. We performed whole-cell patches on two SST interneurons simultaneously (see Material and Methods) and recorded electrical coupling (Fig. 2E). These results showed that electrical synapses are preserved by the NMDG aCSF method and extended the application of NMDG aCSF to the study of gap junctions in slices.

Besides the preservation method, to obtain good-quality brain slices, care must also be taken with the slicing operation. Vertical vibration of the blade, the horizontal amplitude, friction of the blade surface, cutting speed, and the blocking of the brain are all important factors contributing to the quality of slices[18]. In our experiments, we used a Leica VT1200S with vibrocheck which minimizes the vertical vibration of the blade at a customized horizontal amplitude. Generally, razors with low friction are recommended. In the experiments described here, we used razors from Schick (Germany). There are also other options for blades, such as homemade glass, sapphire, or self-customized zirconium ceramic, but they are either difficult to produce or have a low cost-effectiveness. For coronal sections from the prefrontal cortex, we preferred to use a speed of 0.2 mm/s, a horizontal amplitude of 1–1.5 mm, and a 15° blade angle, producing good morphological and functional preservation of cells. For coronal slices from the midbrain, we preferred a speed of 0.07–0.08 mm/s due to the presence of highly myelinated fiber tracts. However, there are no universal settings for the configuration of a vibratome since they vary depending on the specific type of machine and the specific brain region. For example, Guoping Feng and colleagues prefer the VF-200 vibratome from Precisionary Instruments, which has a fixed horizontal amplitude of 2 mm and a 13° blade angle[3,4]. Generally, it is suggested to use a large horizontal amplitude with the vertical vibration at its minimum value (0 mm is best, if possible)[18]. As indicated by Jonathan Ting, if NMDG aCSF is applied during the cardiovascular perfusion, there is no need to worry about the slicing speed and the transfer of slices to the recovery chamber (www.brainslicemethods.com). However, there are still challenges. For example, it is unclear whether the NMDG-based aCSF method is applicable to slices from damaged brain tissue. In addition, the NMDG aCSF method has a limitation on the age of mice, as it is not suitable for mice younger than 30 days. For these mice, the sucrose aCSF or the Tris -based aCSF method is suggested.

Taken together, we showed that the improved NMDG aCSF method offers a better way to perform patch-clamp recordings on neurons compared with the traditional sucrose method. Our results further demonstrated that this method is suitable for studying gap junctions, providing

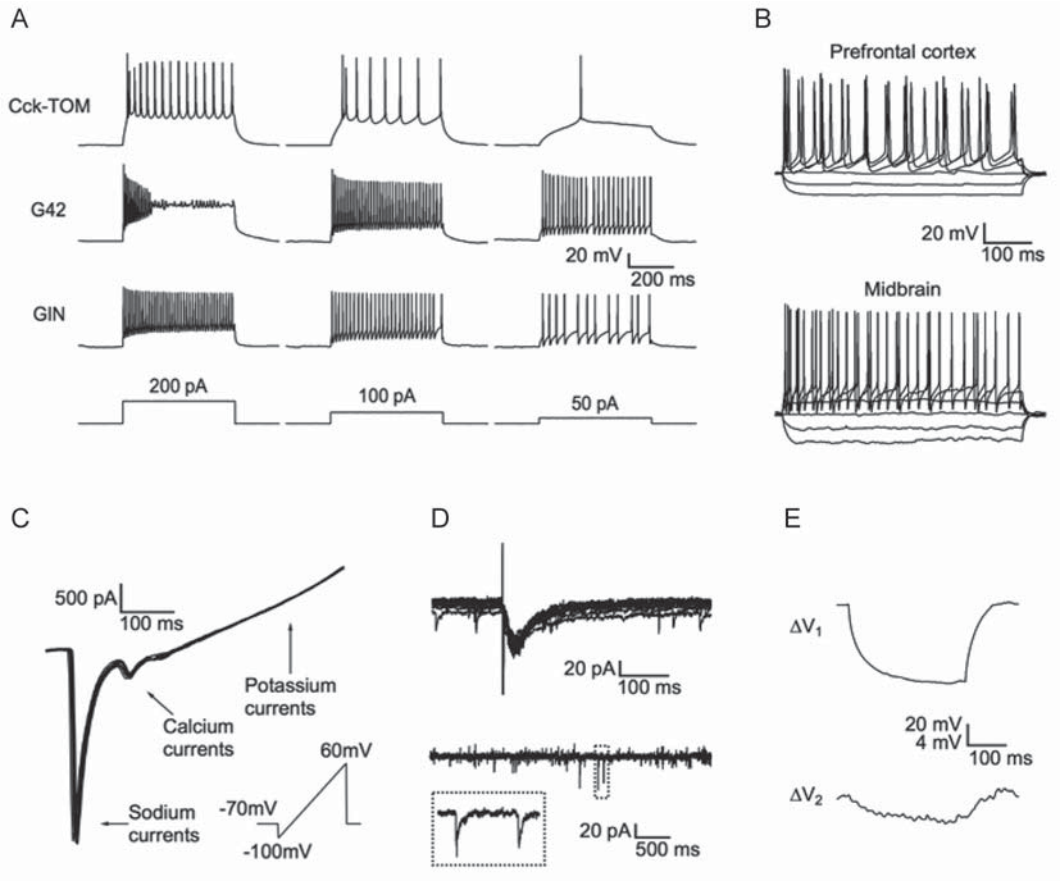

Fig. 2. Electrophysiological properties of neurons in brain slices. Neurons prepared with the NMDG aCSF method from (A) three transgenic mice (3–6 months old) and (B) at two locations (prefrontal cortex and midbrain) all displayed normal firing patterns. (C) Neurons expressed Na^+, Ca^{2+}, and K^+ currents evoked by voltage ramp stimulation from −100 mV to +60 mV, indicating the preservation of these ion channels on the cell membrane. (D) Chemical synapse activity induced by field stimulation (upper panel): a neuron at CA1 was patched while Shaffer collaterals were stimulated by square-wave pulses (square-wave pulses of 100 mV, 1000 Hz, duration 1 ms, the stimulating electrode was >50 μm apart from the recording pipette); and by miniature postsynaptic currents (lower panel): a cell in the CA1 region was held at −70 mV and representative inward traces are shown in the dashed rectangle. (E) Electrical coupling between two somatostatin interneurons (<20 μm apart) in a slice from a mouse ~2 months old. The cells were held at −70 mV with current clamp. Hyperpolarization was generated in one cell (ΔV_1) while voltage deflection was recorded from the other cell (ΔV_2).

an additional method for comparative research studies. However, this work only focused on the presence of chemical and electrical coupling. Whether and how the NMDG aCSF method is able to regulate the chemical and/or electrical coupling ratio remain to be determined.

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·Letter to the Editor·

Dose-dependent regulation of oligodendrocyte specification by β-catenin signaling

Dear Editor,

Although various components of the Wnt/β-catenin pathway have been investigated, there are conflicting reports on the roles of Wnt/β-catenin signaling in oligodendrogenesis and differentiation. For instance, the ΔExon3 mutation of β-catenin[1-4], which stabilizes β-catenin by deletion of the phosphorylation site for the destruction complex, significantly inhibits the differentiation of oligodendrocytes, but knockout of β-catenin also delays it[4]. In addition, overexpression of dominant-negative (dn) forms of *Tcf/Lef* increases the number of oligodendrocyte progenitors (OLPs)[3, 5], and knockout of *Tcf7l2* (also known as *Tcf4*) impairs myelin formation[3, 6]. In contrast, another study showed that overexpression of dn*Tcf7l2* decreases the number of OLPs[7].

Although some of these conflicts could be explained by the finding that Wnt/β-catenin signaling regulates oligodendrocyte development in a stage- and region-specific manner[4, 8-10], its role in the differentiation of oligodendrocytes remains elusive. To address whether Wnt/β-catenin signaling regulates oligodendrocyte development in a dose-dependent manner, we generated mice with different mutations of β-catenin in early OLPs, β-catenin$^{\Delta Exon3/+}$, β-catenin$^{\Delta Exon2\text{-}6/\Delta Exon2\text{-}6}$, and β-catenin$^{\Delta Exon3/\Delta Exon2\text{-}6}$. Quantitative reverse-transcription PCR showed that the dose of Wnt/β-catenin signaling in the compound β-catenin$^{\Delta Exon3/\Delta Exon2\text{-}6}$ mice was lower than that in β-catenin$^{\Delta Exon3/+}$ mice but higher than that in the WT (β-catenin$^{+/+}$) (Supplementary information, Fig. S1). For clarity, β-catenin$^{\Delta Exon2\text{-}6/\Delta Exon2\text{-}6}$, β-catenin$^{\Delta Exon3/\Delta Exon2\text{-}6}$, and β-catenin$^{\Delta Exon3/+}$ mice are referred to as Cat^{Low}, Cat^{High}, and Cat^{High+}, respectively. Consistent with previous work[4], *in situ* hybridization showed that during oligodendrogenesis, *Sox10*- and *Pdgfrα*-positive cells were not present in the spinal cord of Cat^{High+} mice at embryonic day 14.5 (E14.5) (Fig. 1A b and f). In contrast, excess *Sox10*- and *Pdgfrα*-positive cells were present in Cat^{Low} mice (Fig. 1A c and g). Interestingly, compared with WT mice, fewer *Sox10*- and *Pdgfrα*-positive cells were produced in the compound Cat^{High} mice (Fig. 1A d and h, and Ca). Together, these results suggested that at the early stage of oligodendrocyte development in the spinal cord, Wnt/β-catenin signaling inhibits OLP specification of neural stem cells by a dose-dependent mechanism.

We next determined whether the number of OLPs returned to normal in the compound Cat^{High} mice. Although fewer *Sox10*-positive cells were observed in the white matter, both Cat^{Low} and Cat^{High} mice contained normal numbers of *Sox10*- and *Pdgfrα*-positive cells in the gray matter of the spinal cord (Fig. 1B c, d, g, and h, and Cb). Considering that *Sox10* is expressed in both OLPs and differentiated OLs, and *Sox10*-positive cells in the white matter represent mature oligodendrocytes, this result indicates that the number of OLPs returns to normal in the Cat^{Low} and Cat^{High} mice in the perinatal stage. In contrast, there were still no detectable *Sox10*- and *Pdgfrα*-positive OLP cells in the spinal cord of Cat^{High+} mice (Fig. 1B b and f), indicating that a higher level of β-catenin activity leads to a stronger inhibition of OLP generation.

To analyze β-catenin function in OLP differentiation, we assessed the differentiation of OLPs in compound β-catenin mutant mice in the perinatal stage. On postnatal day 0 (P0), expression of the mature OL markers *Mbp* and *Plp* was significantly reduced in Cat^{Low} mice compared to controls (Fig. 1B k and o, and Cc), consistent with the previous observation that β-catenin functions to promote OLP differentiation at late embryonic stages. However, expression of *Mbp* and *Plp* was almost completely inhibited in the spinal cord of Cat^{High+} and Cat^{High} mice (Fig. 1B j, n, l, and p, and Cc). These results demonstrated that full-length β-catenin cannot be replaced by β-catenin with the ΔExon3 mutation, suggesting that a dose of β-catenin activity slightly higher than that in the WT inhibits OLP differentiation. Further studies are needed to test the possibilities that full-length β-catenin regulates OLP differentiation partially independent of Wnt signaling, and β-catenin with the ΔExon3 mutation causes a dominant effect of other signal pathways.

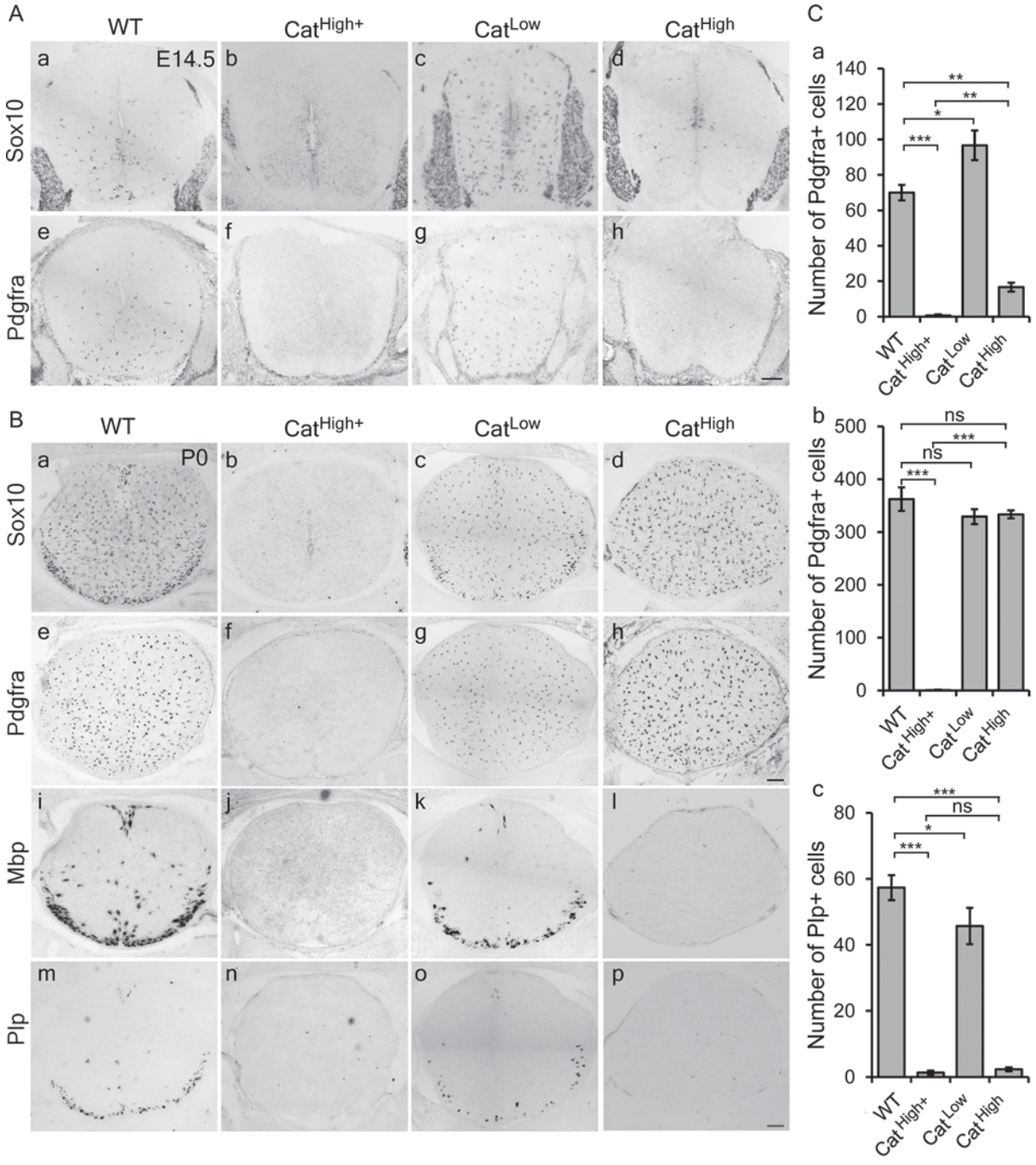

Fig. 1. β-catenin regulates the specification and differentiation of oligodendrocyte progenitors (OLPs). (A) Wnt/β-catenin signaling inhibits the specification of OLPs in a dose-dependent manner. Transverse sections of spinal cord at E14.5 from different β-catenin mutant mice subjected to *in situ* hybridization with *Sox10* (a–d) and *Pdgfrα* (e–h) riboprobes as OLP markers. Cells positive for *Sox10* and *Pdgfrα* were absent, increased, or decreased in the spinal cord from *Olig1*^{Cre}-mediated Cat^{High+}, Cat^{Low}, or Cat^{High} mice, respectively. Scale bars, 100 μm. (B) β-catenin mutations impaire OLP differentiation. All mutants except Cat^{High+} contained normal numbers of cells positive for *Sox10* and *Pdgfrα* at P0 (a–h). Expression of *Mbp* and *Plp* was reduced in Cat^{Low} mice and greatly inhibited in β-catenin^{High+} and β-catenin^{High} mice (i–p). Scale bars, 100 μm. (C) Numbers of *Pdgfrα*$^+$ and *Plp*$^+$ cells per section (mean ± standard deviation of three sections) in the spinal cord of different β-catenin mutant mice at E14.5 (a) and P0 (b and c) ($^*P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.001; ns, not significant; *t*-test).

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