Dysregulation of Protein Kinase A Signaling in the Aged Prefrontal Cortex: New Strategy for Treating Age-Related Cognitive Decline

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Summary

Activation of the cAMP/protein kinase A (PKA) pathway has been proposed as a mechanism for improving age-related cognitive deficits based on studies of hippocampal function. However, normal aging also affects prefrontal cortical cognitive functioning. Here, we report that agents that increase PKA activity impair rather than improve prefrontal cortical function in aged rats and monkeys with prefrontal cortical deficits. Conversely, PKA inhibition ameliorates prefrontal cortical cognitive deficits. Western blot and immunohistochemical analyses of rat brain further indicate that the cAMP/PKA pathway becomes disinhibited in the prefrontal cortex with advancing age. These data demonstrate that PKA inhibition, rather than activation, is the appropriate strategy for restoring prefrontal cortical cognitive abilities in the elderly.

Introduction

The human population’s average life span has increased dramatically over the last century. Consequently, the number of individuals living with diminished cognitive capabilities due to the aging process has also increased. Thus, it is imperative to meet the growing demands for treatments aimed at age-related cognitive decline and to understand fundamental cellular and intracellular changes that emerge in the aging brain (Colombo et al., 1997).

Activation of the cAMP/protein kinase A (PKA) intracellular signaling pathway has been proposed as an avenue for the clinical treatment of age-related deficits in human memory (Barad et al., 1998). Studies of long-term memory consolidation in hippocampus support this idea. Aged animals and humans exhibit deficits in memory consolidation linked to hippocampal function (Gallagher and Rapp, 1997; Herndon et al., 1997). Genetic (Abel et al., 1997; Rotenberg et al., 2000) and pharmacological (Barros et al., 2000; Bernabeu et al., 1997; Bourchoulaizde et al., 1998; Frey et al., 1993) inhibition of PKA (e.g., with Rp-cAMPS) can disrupt long-term potentiation (LTP) in the hippocampus and/or impair long-term memory consolidation, whereas activation of PKA (e.g., with Sp-cAMPS) can induce LTP and enhance memory consolidation (Bernabeu et al., 1997; Frey et al., 1993; Huang and Kandel, 1998). Activation of the cAMP/PKA pathway also enhances long-term memory functions dependent on the amygdala and posterior cortices (Barros et al., 2000; Huang et al., 2000; Schafe and LeDoux, 2000).

PKA activity can also be increased through the systemic administration of agents that inhibit phosphodiesterase (PDE) activity, thus preventing the breakdown of cAMP. Toledo-Morrell first showed that the PDE inhibitor, pentoxifylline, improved hippocampal function in aged rats (de Toledo-Morrell et al., 1984). More recently, research has focused on rolipram, a selective inhibitor of type 4 cyclic AMP phosphodiesterase (PDE4), which elevates levels of cAMP in vivo (Randt et al., 1982). Based on the studies reviewed above, rolipram was tested in animals with long-term memory impairment. Rolipram improved long-term memory consolidation and facilitated LTP in aged mice (Barad et al., 1998) and reversed the memory deficits induced in young rats that were injected with either a muscarinic receptor antagonist (Imanishi et al., 1997; Zhang and O’Donnell, 2000), an NMDA receptor antagonist (Zhang et al., 2000), or a protein synthesis inhibitor (Randt et al., 1982). Thus, there is a highly consistent literature demonstrating that PKA activation enhances the long-term memory functions of posterior cortical and subcortical structures such as the hippocampus. Based on this research in rodents, various pharmaceutical companies are developing agents that enhance PKA activation as a treatment for age-related cognitive decline in humans (Langanreth, 2002).

Although most basic research in rodents has focused on hippocampal mechanisms, deficits in prefrontal cortex function are prominent with advancing age, particularly in human and nonhuman primates. The prefrontal cortex guides behavior and thought using working memory (Goldman-Rakic, 1987) and plays an increasingly important role in the cognitive behavior of primates. The prefrontal cortex is engaged during the retrieval and encoding of memories (especially if it is an effortful process) and is critical for inhibition of proactive interference, for protecting memories and thoughts from distraction, and for allowing us to plan and organize behavior (Bunge et al., 2001; Kapur et al., 1995; Lepage et al., 2000; Stuss and Knight, 2002). Normal aging consistently impairs many of the cognitive functions of the prefrontal cortex in humans, monkeys, and rats (Albert, 1997; Ando and Ohashi, 1991; Bartus et al., 1978; Bimonte et al., 2003; Chao and Knight, 1997; Herndon et al., 1997; Nielsen-Bohlman and Knight, 1995; Rapp and Amaral, 1989; Schacter et al., 1996; West, 1996). Importantly, neuropharmacological studies indicate that the prefrontal cortex is modulated very differently than the hippocampus and thus would require different treatment strategies. For example, in contrast to the hippocampus and posterior cortical areas, infusion of the PKA activator, Sp-cAMPS, directly into the prefrontal cortex impairs working memory function in young adult rats (Taylor et al., 1999).

The current study examined how treatments that alter PKA activation influence prefrontal cortical functions in aged animals. PKA activity was directly activated or...
inhibited in the rat prefrontal cortex using Sp-cAMPS or Rp-cAMPS, respectively, similar to previous studies in young rats (Taylor et al., 1999). Rats were tested on the delayed alternation task in a T-maze, a test of spatial working memory that is impaired by lesions to the pre-limbic/infralimbic prefrontal cortex in rats (Divac, 1971). A parallel study examined the effects of PKA activation in young versus aged monkeys performing a spatial working memory task, delayed response. As Sp-cAMPS and Rp-cAMPS do not penetrate the blood-brain barrier when given systemically, the monkeys were injected with the PDE4 inhibitor, rolipram, similar to previous research in rodents (Barad et al., 1998; Imanishi et al., 1997; Randt et al., 1982; Zhang and O’Donnell, 2000).

Western blot analysis was performed on prefrontal cortex, hippocampus, and cerebellum of aged versus young adult rats to characterize age-related changes in the cAMP-PKA signaling pathway. Two AC isoforms were examined, AC2 and AC3. The regulatory and catalytic subunits of PKA and the phosphodiesterase isoforms PDE4a, PDE4b, and PDE4d were measured, as well as indirect measures of PKA activity, cAMP response element binding protein (CREB), and CRE binding. Activation of the cAMP/PKA pathway has been shown to upregulate CREB expression through CRE binding in a number of cell types (Coven et al., 1998). Finally, phospho-CREB immunohistochemistry was performed on rat prefrontal cortex and hippocampus to provide a more direct measure of PKA activity. The findings from both the behavioral and biochemical studies suggest that the cAMP/PKA pathway becomes disinhibited in the prefrontal cortex with advancing age and contributes to loss of prefrontal cortical cognitive function.

Results

Rats

Young adult (9 months) and aged (approximately 24 months when tested) rats were surgically implanted with guide cannula directed above the prefrontal cortex (Figure 1). Animals were trained on a delayed alternation task in the T-maze, a test of spatial working memory that depends upon the functional integrity of the prefrontal cortex (Divac, 1971). Delays began at “0” seconds (s) and were gradually raised in 5 s intervals throughout the drug study as the animal performed ≥80% correct on consecutive days (see Experimental Procedures). Thus, the number of sessions to achieve a delay of 15 s can be used to characterize the cognitive ability of the animal performing the task. Young adult rats (n = 26) required an average of 26.7 ± 3.7 testing sessions to achieve a 15 s delay, while aged rats (n = 15) required an average of 49.9 ± 8.4 testing sessions (p = 0.004, unpaired t test). There was great variability in this measure within the aged group, with some animals performing as well as the young, and others much impaired (see x axis in Figures 2A and 2B).

Once the rats were performing in a stable manner (see Drug Infusions in Experimental Procedures), PKA signaling in the prefrontal cortex was manipulated prior to testing by infusing either the PKA activator Sp-cAMPS or the PKA inhibitor Rp-cAMPS into the prefrontal cortex. Results were compared to vehicle infusion. The Sp-cAMPS dose was selected based on previous work in young rats, which had shown that 21 nmol/0.5 μl Sp-cAMPS significantly impaired performance, whereas 0.21 and 2.1 nmol/0.5 μl had no effect (Taylor et al., 1999). The Rp-cAMPS dose was based on a pilot study, in which young and aged rats were examined after a wide range of Rp-cAMPS concentrations (0.021, 0.21, 2.1, 21, 42, and 84 nmol/0.5 μl). In young rats, no dose of Rp-cAMPS had any effect, with the exception that impairment was found at the highest dose (84 nmol: 51.9% ± 7% correct, p = 0.031 compared to vehicle). Seizures were observed in some animals following this high dosage; thus, the relevance to cognitive function remains in question. No dose of Rp-cAMPS impaired the aged rats, including the 84 nmol dosage (62.5% ± 7.3% correct, p = 0.45 compared to vehicle). Pilot studies indicated that the 2.1 nmol/0.5 μl Rp-cAMPS dose improved performance in aged rats; thus, this dose became the focus of the current study (see below). Given the fragility of the aged animals and thus the limited number of infusions that could be done in this age group, we focused on the 2.1 nmol/0.5 μl dose for both Rp-cAMPS and Sp-cAMPS in the current research.

The effects of 2.1 nmol/0.5 μl Sp-cAMPS and 2.1 nmol/0.5 μl Rp-cAMPS in aged rats depended upon the cognitive status of the animal (Figures 2A and 2B). As shown in Figure 2A, the greater the number of test sessions needed to achieve a 15 s delay, the greater the impairment following infusion of Sp-cAMPS into the prefrontal cortex. In other words, the greater the cognitive deficit
due to age, the more impaired the rat was during the testing session following an acute activation of PKA (significant correlation between days to achieve 15 s delay and response to Sp-cAMPS: \( r = -0.78; p < 0.01 \), Pearson’s test). Conversely, the greater the cognitive deficit due to age, the more the rats were improved by PKA inhibition with Rp-cAMPS (\( r = 0.70; p < 0.01 \), Figure 2B). Therefore, the aged animals that needed prefrontal cortical cognitive enhancement the most were improved by inhibition rather than activation of the PKA pathway.

This effect was not simply an artifact of the delay length used in the task, as Rp-cAMPS did not improve the aged, cognitively intact rats even when it was administered when delay intervals were short. Correlational analyses showed no significant relationship between delay interval and response to either drug. Rather, the response to the drug depended on the overall prefrontal cortical abilities of the aged rat. In contrast to aged animals, there was no correlation between cognitive status and the response to 2.1 nmol Rp-cAMPS (\( r = -0.065, \text{ns} \)) or 2.1 nmol/0.5 \( \mu l \) Sp-cAMPS (\( r = 0.21, \text{ns} \)) in young adult rats. Interestingly, in aged rats there was a significant correlation between the degree of impairment induced by 2.1 nmol/0.5 \( \mu l \) Sp-cAMPS and the amount of improvement seen when infusing 2.1 nmol/0.5 \( \mu l \) Rp-cAMPS in the same aged animal (\( n = 11 \), Pearson’s test: \( r = -0.76, p < 0.01 \), Figure 2C). In other words, the more an aged rat was impaired by Sp-cAMPS, the more it was improved by Rp-cAMPS. These results contrast with studies in the hippocampus and posterior cortex, where Sp-cAMPS improves rather than impairs memory performance, providing more support for the idea that the prefrontal cortex is regulated differently than other brain regions.

Monkeys

Sp-cAMPS and Rp-cAMPS do not cross the blood-brain barrier, and thus cannot be administered systemically. However, the PDE4 inhibitor, rolipram, is capable of penetrating the brain following systemic administration and has been shown to enhance long-term memory consolidation in aged mice (Barad et al., 1998). Thus, rolipram was administered to young adult (4–18 years, \( n = 7 \)) and aged (18–30 years, \( n = 7 \)) rhesus monkeys (Barts et al., 1978; Rapp and Amaral, 1989). Within each test session, performance was assessed across a variety of delay lengths, ranging between a 0 s delay control condition and the delay where each monkey performed at chance (50%). Three intermediate delay intervals were also used so that drug effects could be examined across the span of each monkey’s abilities. A wide rolipram dose range was examined in all animals: 0, 0.01, 0.1, 1.0, 10.0, 50, or 100 \( \mu g / kg \) was administered in random order with at least 1 week washout between doses. As side effects of agitation and drooling were observed at doses \( \geq 50 \mu g / kg \), only the 0, 0.01, 0.1, 1.0, and 10.0 \( \mu g / kg \) doses were used for analyses of effects on cognitive performance.

Rolipram had a significant effect on delayed response performance (significant within subjects effects of rolipram: \( F(4,48) = 3.42, p = 0.015 \)), but its effects on performance were dependent upon the age of the animal [significant between subjects effects of age: \( F(1,12) = 4.99, p = 0.045 \)]. As can be seen in Figure 3A, young monkeys tended to be improved by the 0.1 \( \mu g / kg \) dose of rolipram \( [F(1,6) = 4.94, p = 0.068] \). However, this finding did not reach statistical significance due to the great variability in individual response to rolipram (SEM = 4.7% for rolip-
rolipram’s effects on performance at each delay length confirmed this interpretation (effect of delay F(4,20) = 5.048, p = 0.006; effect of rolipram F(1,5) = 12.411, p = 0.017). Rolipram did not increase errors following the 0 s delay control condition F(1,5) = 1.840, p = 0.233) but did impair performance following intermediate delays when the prefrontal cortex was needed to guide behavior [F(1,5) = 8.67, p = 0.032 and F(1,5) = 15.21, p = 0.011]. There was no further impairment by rolipram at the longest delays, as the aged monkeys were performing at chance levels under these conditions following saline administration [F(1,5) = 0.044, p = 0.840]. The interaction of advancing age with response to rolipram was also evident in a correlational analysis: there was a significant correlation between estimated age of the monkey and the degree of impairment induced by 10.0 μg/kg rolipram (Figure 3C; Spearman test: r = 0.579, p = 0.03). Thus, in contrast to rolipram’s beneficial influence on hippocampal-dependent memory functions in aged mice, rolipram impaired working memory performance in aged monkeys.

Age-Related Changes in the PKA Pathway

In an effort to understand the underlying mechanisms involved in the differential response to PKA manipulations with age, young versus aged rat brains were compared using Western analyses and immunohistochemistry. Three regions of the brain associated with learning and memory were examined: the prefrontal cortex, hippocampus, and cerebellum. Basal PKA levels (regulatory or catalytic subunit immunoreactivity in all brain regions examined) were not affected by age (data not shown). Similarly, there were no age-related changes in PDE4a, PDE4b, or PDE4d (data not shown). Previous evidence had suggested that the AC system, which is upstream of PKA, may be more vulnerable to age, particularly in the cerebral cortex, hippocampus, and cerebellum (Araki et al., 1995, 1997). Therefore, immunoblot assays examined basal levels of AC2 and AC3 isoforms in young versus aged rats (Table 1). Proteins downstream of cAMP/PKA signaling, CREB and CRE binding, were also assessed as an indirect measure of PKA activity. Finally, a separate immunohistochemical study assessed numbers of phospho-CREB-positive cells in prefrontal cortex and hippocampus as a direct measure of PKA activity in young versus aged rats performing a working memory task.

In the prefrontal cortex, AC3 tended to be reduced but was not significant due to the high degree of variability in the young animals (p = 0.2; Table 1). No significant reduction in AC2 was found (p = 0.67; Table 1). As shown in Figure 4A, CREB immunoreactivity tended to be increased (p = 0.08) and CRE binding was significantly higher in the aged prefrontal cortex (p = 0.006).

In the hippocampus, AC3 immunoreactivity was significantly reduced in aged versus young rats (p = 0.02; Table 1). As in the prefrontal cortex, there was no change in AC2 (p = 0.56; Table 1). However, unlike the prefrontal cortex, there was no change in CREB levels (p = 0.74) or in the amount of CRE binding (p = 0.26) (Figure 4A).

Finally, in the cerebellum of aged rats, there was no change found in the levels of AC isoforms (p > 0.7; Table 1). Similar to the hippocampus, there was no change in
Table 1. Basal Levels of Adenylyl Cyclase Isoforms AC2 and AC3 in Young versus Aged Rats as Percent of Young Controls

<table>
<thead>
<tr>
<th>AC Isoform</th>
<th>Prefrontal Cortex</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
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<tr>
<td></td>
<td>Young (n=6)</td>
<td>Aged (n=6)</td>
<td>Young (n=6)</td>
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<tr>
<td>AC2</td>
<td>100 ± 15a</td>
<td>90 ± 18</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>AC3</td>
<td>100 ± 11</td>
<td>81 ± 6</td>
<td>100 ± 7</td>
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*Data presented as mean ± SEM.

CREB levels (p = 0.35) or in the amount of CRE binding (p = 0.9) (Figure 4A). Thus, none of the measures were different in the aged cerebellum.

Overall, these results suggest that the prefrontal cortex may be affected differently than these other brain regions by the aging process, as evidenced by the increased CRE binding and trend for increased CREB levels in aged prefrontal cortex tissue. The increase in CRE binding may indirectly reflect increased PKA activation since activation of this pathway has been shown to upregulate CRE-mediated gene expression including the CREB gene itself (Coven et al., 1998).

The catalytic subunit of PKA directly phosphorylates CREB in the nucleus (Gonzalez and Montminy, 1989). As levels of phospho-CREB are a direct measure of PKA activity, we examined the number of cells staining for phospho-CREB in aged versus young rats performing the delayed alternation task immediately prior to sacrifice. All but one of the aged rats showed impaired performance on the delayed alternation task, achieving an average delay of only 3.3 s at time of sacrifice. As is shown in Figure 5, aged rat prefrontal cortical tissue had a significantly higher number of cells immunostained for phospho-CREB compared to young prefrontal cortex (p = 0.003, unpaired t test). Furthermore, the intensity of the staining (indicative of the amount of phospho-CREB present within each cell) was higher in the aged than in the young prefrontal cortex (Figure 6, upper panel). In contrast, aged hippocampal tissue had a slightly lower number of cells that were phospho-CREB immunoreactive (Figure 5), but this difference was not significant (mean aged = 154.4; mean young = 161.4; p = 0.553). The cells from aged hippocampal sections stained less darkly than the cells from the young hippocampus (Figure 6, lower panel), consistent with less PKA activity within each cell in the aged hippocampus. Thus, the prefrontal cortex and hippocampus showed opposite profiles with advancing age: the prefrontal cortex showed indices of increased PKA activity, while the hippocampus exhibited evidence of decreased PKA activity.

Discussion

The working memory functions of the prefrontal cortex are regulated differently than hippocampus and posterior cortical regions. Thus, activation of intracellular signaling pathways that strengthen memory consolidation in hippocampus impair the working memory functions of the prefrontal cortex (Birnbaum et al., 1999; Taylor et al., 1999). For example, infusions of the PKA activator, Sp-cAMPS, into the hippocampus enhances memory consolidation (Bernabeu et al., 1997), while infusion in the prefrontal cortex impairs working memory performance (Taylor et al., 1999). In the hippocampus, PKA activation is thought to keep memory fixed and enhance long-term storage of information. In contrast, the prefrontal cortex may be especially vulnerable to disinhibition of the PKA pathway, given the great need for erasure in the updating of working memory. Until now, research has focused on activating PKA to strengthen hippocampal function and improve memory in aged subjects. However, no research has examined the consequences of activating PKA in the prefrontal cortex with advancing age, despite the prevalence of prefrontal cortical cognitive deficits in the elderly.
hippocampus. As all but one of the aged rats showed impaired performance on the delayed alternation task, future studies will be needed to compare phospho-CREB staining in cognitively intact versus impaired aged rats. Taken together, these data indicate that PKA signaling in the prefrontal cortex becomes disinhibited with advancing age and that further stimulation of this pathway, even with a very low dose of an activator such as Sp-cAMPS, exacerbates cognitive deficits. Conversely, a very low dose of the PKA inhibitor, Rp-cAMPS, was sufficient to ameliorate cognitive impairment in aged rats. These data indicate that the cAMP/PKA signaling pathway becomes disinhibited within prefrontal cortical neurons with advancing age and that relatively subtle manipulations of this pathway can have marked effects on prefrontal cortical cognitive functioning in aged animals.

The results in monkeys provide additional support for this hypothesis and extend these findings to primates. Systemic administration of rolipram, which indirectly increases PKA activity, had little effect in young monkeys but impaired the prefrontal cortical cognitive performance of aged monkeys following the 10 μg/kg dose. The detrimental effects of rolipram in the aged monkeys appeared to represent deficits in prefrontal cortical cognitive function rather than nonspecific impairment due to side effects, as there was no increase in errors under 0 s delay control conditions and no change in behavioral ratings at this dose. Interestingly, the magnitude of the impairment correlated with increasing age, consistent with progressive disinhibition of PKA signaling with advancing age. These results in aged monkeys are opposite to what was found with rolipram administration in aged mice, who were improved following systemic rolipram administration (Barad et al., 1998). This discrepancy was likely due to the fact that the mice were per-
forming a task dependent on the hippocampus, while the aged monkeys were performing a task dependent on the prefrontal cortex. The current results demonstrate that indirect activation of the cAMP/PKA intracellular pathway impairs rather than improves the cognitive functioning of the prefrontal cortex in aged monkeys with prefrontal cortical deficits. Therefore, cAMP/PKA activation is not an appropriate strategy for treating age-related cognitive decline. Interestingly, the rolipram dose that impaired prefrontal cortical function in our aged monkeys (10 μg/kg) is within the dose range of rolipram tried as an experimental antidepressant in young adult humans (Zhu et al., 2001). The current data caution that use of this dose range in elderly humans may worsen prefrontal cortical regulation of thought and affect. Thus, an exclusive focus on hippocampal mechanisms may have hazardous consequences when treating age-related cognitive decline in humans.

The present study may also explain why α2 adrenergic agonists are so effective in enhancing prefrontal cortical cognitive function in aged animals. Studies in both rodents and monkeys have found that α2 adrenergic agonists improve prefrontal cortical but not nonprefrontal cortical cognitive functions and that these beneficial effects become increasingly prominent with advancing age (Arnsten and Goldman-Rakic, 1985; Carlson et al., 1992; Franowicz and Arnsten, 1998; Rama et al., 1996; Tanila et al., 1996). It is well established that α2 adreceptors couple to G proteins and reduce PKA activation. The current data suggest that aged animals have excessive PKA activity in the prefrontal cortex and thus may show greater benefit from PKA inhibition via α2 adreceptors stimulation.

In contrast to the prefrontal cortex, hippocampal memory functions are impaired by stimulation of α2 adrenoceptors (Tanila, 2001) and enhanced by activation of the cAMP/PKA pathway. For example, memory consolidation and LTP (late phase) depend on activation of cAMP/PKA signaling (Abel et al., 1997; Barros et al., 2000; Bernabeu et al., 1997; Bourchouchadze et al., 1998; Frey et al., 1993; Huang and Kandel, 1998; Huang et al., 2000; Rotenberg et al., 2000; Schafe and LeDoux, 2000). Thus, changes in the PKA pathway with advancing age in hippocampal tissue may contribute to impaired memory consolidation. Previous studies have shown that forskolin binding is reduced with age in various brain regions including the cerebral cortex and hippocampus, suggesting that the adenylyl cyclase system and thus the PKA pathway are affected by the aging process (Araki et al., 1998, 1997). Our results elaborated on these findings, observing reductions in AC3 levels in the aged hippocampus. Since adenylyl cyclases are often concentrated within spines (Mons et al., 1995) and spines are lost with age (Page et al., 2002), isoforms such as AC3 may be in a particularly vulnerable location. However, it is not yet clear if reduced levels of AC3 contributes to the decrease in phospho-CREB staining in the aged hippocampus.

Spines are also lost from prefrontal cortical pyramidal neurons with advancing age (Feldman and Dowd, 1975; Page et al., 2002); thus, slight reductions in AC3 in prefrontal cortex may also result from subtle degenerative processes during aging. Although there was little effect of age on AC2 and AC3 in the aged prefrontal cortex, future studies should measure additional AC isoforms to understand what is causing this PKA overactivation. It is possible that the activity of other AC isoforms is increased or that there is a loss of some inhibitory component in the pathway. Interestingly, AC2, which is unchanged with age, is under little inhibitory regulation and may have the capacity to be superstimulated, e.g., by βγ subunits (Baker et al., 1999; Iyengar, 1993; Taussig et al., 1994). Thus, it is plausible that unregulated AC2 activity contributes to excessive PKA activity in the aged prefrontal cortex. Future research may also wish to examine age-related changes in other mechanisms that regulate PKA activity. For example, alterations in the ubiquitin-proteasome cascade, which can lead to persistent PKA activation (Lopez-Salon et al., 2001), might play a role in this age-related, prefrontal PKA overactivation.

The disinhibition of PKA signaling in the aged prefrontal cortex may also be related to a history of stress exposure. In young adult animals, acute exposure to uncontrollable stress rapidly impairs prefrontal cortical cognitive function, a mechanism that may have survival value under dangerous conditions (Arnsten, 1998). Stress-induced prefrontal cortex dysfunction results from high levels of catecholamine release in the prefrontal cortex that engage detrimental cAMP/PKA actions (reviewed in Arnsten, 2000). Glucocorticoids released during stress also impact on prefrontal cortical function. For example, chronic exposure to high levels of corticosterone induces spine loss from prefrontal cortical neurons in young adult rats (Wellman, 2001), similar to that observed in aged animals and humans (Feldman and Dowd, 1975; Page et al., 2002). Given that some AC isoforms have been localized to dendritic spines (Mons et al., 1995), chronic stress exposure may induce architectural changes that impact on cAMP/PKA signaling. These data suggest that there may be a relationship between stress exposure over the lifespan and disinhibition of PKA signaling in the aged prefrontal cortex. Information regarding the history of stress exposure (e.g., subordinate versus dominant relationship with cage mates) for the aged rats in the current study is not known. Future research could examine the relationship between a history of stress exposure, disinhibition of PKA signaling, and erosion of prefrontal cortical cognitive function with advancing age.

In summary, the aging process does not effect all brain regions in the same manner. In the hippocampus, there is a simple reduction in cAMP/PKA signaling with advancing age that weakens memory consolidation. This contrasts with the prefrontal cortex, where advancing age unleashes detrimental PKA actions that impair working memory. The current study revealed that activating PKA, either directly with Sp-cAMPS or indirectly with rolipram, exacerbated working memory deficits, while PKA inhibition with Rp-cAMPS strengthened working memory performance. Thus, inhibiting the cAMP/PKA activity appears to be the more appropriate strategy for treating prefrontal cortical cognitive dysfunction in the elderly. As the prefrontal cortex plays an expansive role in human cognitive behavior, the distinct neurochemical needs of this cortex must be respected for the development of effective cognitive enhancers.
Experimental Procedures

All procedures were approved by the Yale Institutional Animal Care & Use Committee. Care of the rats and monkeys followed the guidelines in “Guide for the Care and Use of Laboratory Animals.”

Rat Studies

Subjects
Aged (20 months) and young (9 months) male, retired breeder Sprague Dawley rats from Harlan (Indianapolis, IN) were single-housed in filter frame cages. Aged rats were approximately 24 months old upon initiation of pharmacological testing. Animals were on a 12-hour light/dark cycle, and experiments were conducted during the light phase. Rats were slowly habituated to a restricted diet (16 gm/day per rat) of autoclaved Purina (St. Louis, MO) rat chow during the first two weeks. Food was given immediately after behavioral testing and water was available ad libitum. Rats were weighed weekly to confirm that they were not undergoing irregular weight loss due to regulated diet. Food rewards during cognitive testing were highly palatable miniature chocolate chips. Rats were assigned a single experimenter who handled them extensively before behavioral testing.

Delayed Alternation in T-Maze

Rats were habituated to a T-maze (dimensions, 90 × 65 cm) until they were readily eating chocolate chips placed at the end of each arm and were acclimated to handling. After habituation, rats were trained on the delayed alternation task. On the first trial, animals were rewarded for entering either arm. Thereafter, for a total of 10 trials per session, rats were rewarded only if they entered the maze arm that was not previously chosen. Between trials the choice point was wiped with alcohol to remove any olfactory clues. The delay between trials started at “0” s (i.e., about 1.5 s, minimum possible for delayed alternation) and was subsequently raised in 5 s intervals as needed to maintain performance at 60% to 70% correct. Thus, delay achieved in a set number of testing sessions can be used as an index of cognitive ability.

Canulae Implantation

After training on the delayed alternation task, animals underwent stereotaxic implantation of chronic guide canulae as described previously (Taylor et al., 1999). Guide canulae (Plastics One; 2.8 mm) with stylettes were aimed dorsal to the medial prefrontal cortex (prelimbic prefrontal cortex; stereotaxic coordinates: anterior-posterior, +3.2 mm; mediolateral, ±0.75 mm; dorsoventral, stylet reaching to −4.2 mm) as shown in Figure 1. Due to the rats’ age and fragility, surgery was performed under low doses of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) injected (i.p.) prior to the start of the procedure. These agents were supplemented with gas anesthesia (isoflurane) administered during surgery via nosecone. Sterile stylettes were inserted in the cannula to maintain patency. Great care was taken to minimize pain and infection postoperatively to decrease stress to the animal. The region surrounding the cemented guide cannula was treated with triple antibiotic and cleaned daily if needed for a period of about a week. Animals were also acutely treated with Buprenex (0.01 mg/kg) to decrease pain.

Drug Infusions

Rats were initially adapted to a mock infusion protocol to minimize any stress associated with the procedure. Rats were gently restrained while the stylettes were removed and replaced with 30 gauge sterile infusion needles that extended to 4.5 mm doroventral below the skull. Bilateral infusions were driven by a Harvard Apparatus (Holliston, MA) syringe pump set at a flow rate of 0.25 μl/min using 25 μl Hamilton syringes for an infusion time of 2 min. Needles remained inserted in place for 2 min after completion of the infusion. Stylettes were inserted back into the cannulae, and behavioral testing was performed immediately after the infusion procedure. Drug treatments and vehicle were administered in a counterbalanced order with at least 1 week between each infusion. Counterbalancing ensured that rats received drug infusions both early in the study when delays were short, as well as later in the study when delays were longer. Animals were required to exhibit stable baseline performances (2 consecutive test sessions of 66–70% correct) prior to drug administration. Sp-cAMPS and Rp-cAMPS were purchased from Sigma RBI (St. Louis, MO). Sp-cAMPS and Rp-cAMPS were dissolved in sterile PBS as described previously (Punch et al., 1997). The experimenter testing the animal was unaware of drug treatment conditions.

Histology

Rats were administered an overdose of ketamine and xylazine and sacrificed by decapitation. Brains were removed, stored in formalin, sectioned, and analyzed for histological verification of cannulae placement. All rats had correctly placed cannulae (Figure 1).

Data Analysis

The relationship between efficacy of Sp-cAMPS or Rp-cAMPS in aged rats versus cognitive status of the aged animals was assessed using a Pearson’s r test. The factors were percent correct on the delayed alternation task following drug treatment, versus the number of testing sessions required to achieve a 15 s delay. The relationship between the efficacy of Sp-cAMPS versus Rp-cAMPS in the same aged rat was also assessed using a Pearson’s r test. The factors were percent correct on the delayed alternation task following Sp-cAMPS treatment versus the same animal’s performance under Rp-cAMPS during a different testing session.

Monkey Studies

Subjects
The animals used in this study were rhesus monkeys (Macaca mulatta) ranging in age from 4 years (postpubescent) to over 30 years. Actual birthdates were not available for several of the aged animals who were wild caught. Ages were estimated by the veterinarians based on health records, teeth, and known history; several had been in the Yale colony for more than 15 years. The young adult monkeys (4–18 years) consisted of 6 females and 1 male; the aged monkeys (15–30 years, n = 7) were all female. The monkeys were individually housed and maintained on a diet of Purina monkey chow supplemented with fruit. Animals were always tested at the same time of day immediately prior to feeding. Highly palatable food rewards (e.g., peanuts, raisins, or chocolate chips) were utilized during testing to minimize the need for dietary regulation.

Delayed Response Testing

Cognitive testing occurred in a Wisconsin General Testing Apparatus (WGTA) situated in a sound-attenuating room. Background masking noise (60 dB, wideband) was also used to minimize auditory distractions. Each monkey was assigned to a single experimenter who knew the animal well but was unaware of the drug treatment conditions. The animals were tested twice a week with 3–4 days separating each test session (e.g., Monday and Thursday). The monkeys had been previously trained on the spatial delayed response task as described (Arnst et al., 1988). During delayed response, the animal watched as the experimenter baited one of several foodwells with a food reward. The number of foodwells varied from two to four wells, depending on the monkey’s performance level and experience with the task. Care was taken by the experimenter to ensure that the animal attended the baiting procedure. The foodwells were then covered with identical cardboard plaques, and an opaque screen was lowered between the animal and the foodwells for a specified delay. At the end of the delay, the screen was raised and the animal was allowed to choose. Reward was quasi-randomly distributed between the left and right wells over the 30 trials that made up a daily test session. Five different delay lengths (referred to as delays A through E) were quasi-randomly distributed over these 30 trials. The shortest of these delays was less than 1 s (the “0” s A delay). The remaining delays were in the range that for each individual monkey yielded baseline performance of about 70% across all delays (i.e., 18–22 trials correct of the possible 30 trials). For example, the delays for one animal might be A = 0, B = 5, C = 10, D = 15, and E = 20 s. The “E” delay for the 7 young monkeys ranged between 20 and 80 s; for the aged monkeys it ranged from 12 to 80 s. Please note that because the monkeys in this study have participated in research for varying amounts of time (some monkeys as long as 15 years, others for only three years), the “delay achieved” measure cannot be used as an accurate assessment of prefrontal cortical function in monkeys as it can in rats, who were all tested for equal amounts of time.

Sedation Assessment

During each cognitive testing session, the experimenter rated the animal’s level of sedation/agitation and aggression on rating scales.
Sedation and agitation were rated using a 9 point scale, where 9 = too sedated to test; 3 = too agitated to test; 3 = agitation that interferes with testing, 2 = slight agitation that does not interfere with testing, 1 = more alert than usual, 0 = normal level of arousal, 1 = quieter than usual, 2 = sedated (drooping eyelids, slowed movements), 3 = intermittent sleeping, and 4 = too sedated to test. Assocation was rated using a similar scale, where 0 = dramatically more aggressive, 1 = moderately more aggressive, 2 = normal, 3 = mildly less aggressive, 2 = moderately less aggressive, and 3 = dramatically less aggressive.

Data Administration
Rolloipram (purchased from Sigma R1, St. Louis, MO) was dissolved in 0.2 ml 100% ethanol and 0.8 ml sterile saline and diluted with saline to the appropriate concentration for the following doses: 0.01, 0.1, 1.0, 10.0, 50, or 100 μg/kg. Drug solutions were made up fresh each day under sterile conditions. Drug or vehicle was injected intramuscularly 1 hr prior to cognitive testing. The order of dose administration was determined quasi-randomly, and the experimenter testing the animal was unaware of the treatment condition. A washout period of at least 10 days occurred between drug treatments. Monkeys were required to return to stable baseline performance for 2 consecutive testing days prior to new drug treatment. Given these prolonged washout conditions, the research took approximately 18 months to complete.

Data Analysis
The rolipram data from young versus aged monkeys were analyzed using a two-way analysis of variance (2-ANOVA-R) with a within subjects factor of rolipram dosage and a between subjects factor of age. Planned comparison (test of effects) examined the effects of specific rolipram doses versus saline for the young or aged monkeys. A more detailed analysis of the effects of 10 μg/kg rolipram at each delay interval was performed on the data from the aged monkeys (2-ANOVA-R; within subjects factors of rolipram and delay interval). Correlations between estimated age of the monkey and the response to rolipram were performed using a nonparametric Spearman test, as actual birthdates were only known for a subset of animals.

Biochemistry of Rat Brain
Subjects
Young adult (9 months, n = 6) and aged (24 months, n = 6) male, retired breeder Sprague Dawley rats were purchased from Harlan (Indianapolis, IN) and single-housed in filter frame cages under standard laboratory conditions as described above. Aged animals were sacrificed by decapitation at the age of 24 months for biochemi- cal analysis.

Immunoblotting of Adenylyl Cyclase Isoforms and CREB
Extracts of prefrontal cortex, hippocampus, or cerebellum from 9- or 24-month-old animals were prepared for immunoblot analysis as previously described (Lane-Ladd et al., 1997; Sakai et al., 2002). In most cases, the tissue samples were homogenized (10 mg of wet weight/ml) in 1% SDS. Aliquots of the homogenates were subjected to SDS-PAGE and transferred to nitrocellulose filters for immu noblotting. Rabbit polyclonal anti-adenyl cyclase types II and III, R.S.D. and MERIT Award AG06036 to A.F.T.A. Araki, T., Kato, H., Fujiwara, T., and Itoyama, Y. (1995). Age-related changes of working and reference memory in the rat. Neurosci. Lett. 128, 172–20.

References


