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Review

The cyclic AMP phenotype of fragile X and autism

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ABSTRACT

Cyclic AMP (cAMP) is a second messenger involved in many processes including mnemonic processing and anxiety. Memory deficits and anxiety are noted in the phenotype of fragile X (FX), the most common heritable cause of mental retardation and autism. Here we review reported observations of altered cAMP cascade function in FX and autism. Cyclic AMP is a potentially useful biochemical marker to distinguish autism comorbid with FX from autism per se and the cAMP cascade may be a viable therapeutic target for both FX and autism.

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Contents

1. Introduction

Fragile X (FX) syndrome, or Martin-Bell syndrome, is the most common inheritable cause of mental retardation, with a prevalence of 1:2000–4000 for males and in 1:4000–8000 for females (Oostra and Willemsen, 2003; Tsiouris and Brown, 2004). The genotype is a CGG trinucleotide amplification on the X chromosome (Xq27.3) in the 5′ untranslated region of the fragile X mental

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retardation-1 (FMR1) gene that suppresses production of fragile X mental retardation protein (FMRP) (Pieretti et al., 1991; Verkerk et al., 1991; Kaufmann et al., 2002). FX premutation carriers have more than 55 CGG repeats and those with more than 200 repeats have a full FX mutation (Fu et al., 1991). The comorbidity of autism and FX was initially reported to be around 18% (Brown et al., 1982b) and a lower percentage of those with autism have FX (about 7%) (Brown et al., 1982a, 1986; Loesch et al., 2007). Although reported incidences vary, FX is known to be the most common inherited cause of autism. Autism is a behaviorally defined neurodevelopmental disorder with a characteristic triad of deficient diagnostic domains: social, behavioral, and communicative (Rapin, 1997). Anxiety-related social behavior and memory deficits are reported in both the autism (Williams et al., 2006; Gillott and Standen, 2007) and FX behavioral phenotypes (Cornish et al., 2001; Hagerman, 2002). Signaling deficiencies in mGluR and cyclic AMP (cAMP)-mediated mechanisms are central to FX pathophysiology (Berry-Kravis, 1990; Berry-Kravis et al., 1995; Miyashiro and Eberwine, 2004). In this paper, we examine the evidence for the contributions of defects in signaling pathways in FX and autism behavior by reviewing the cAMP cascade and its role in stress, anxiety, and memory and relate these findings to the potential for cAMP-mediated therapy for FX and autism.

2. Cyclic AMP cascade

The cAMP cascade is a ubiquitous, prototypical second messenger signaling system that transduces extracellular neurotransmitter or hormone messages that bind to a receptor. Adenylate cyclase (AC) is an enzyme that catalyzes cAMP formation from ATP in the presence of Mg^{2+}. Of the 10 adenylate cyclase isozymes, all membrane-bound AC isoforms (AC1-9) are expressed in the brain. Their regional distribution is isoform-dependent (Chern, 2000) and their regulation is complex and isozyme-specific (Tang and Hurley, 1998). Serotonin, adrenergic, dopaminergic, adenosine, vasoactive intestinal peptide, muscarinic, GABA, and opiod receptors, among others, signal through the cAMP cascade (Lauder, 1993) via specific heterotrimeric G proteins. G proteins are guanine nucleotide binding proteins that act as signal transducers by mediating the signal of receptor activation across the membrane to effectors, like adenylate cyclase, that can alter levels of second messengers, like cAMP. Each G protein is a heterotrimer consisting of three subunits: α, β, γ. Based on α subunit similarity, there are four families of G proteins: Gs, Gi, Gq, and G_{12} (Cabrera-Vera et al., 2003). Gs activates all membrane-bound AC isoforms and forskolin, an AC activator (Seamon et al., 1981), is able to activate all but AC9 (Cumbay and Watts, 2004). Gi regulates cAMP levels through inhibition of adenylate cyclase and Gq activates phospholipase C to cleave phosphatidylinositol bisphosphate (PIP2) into both inositol triphosphate (IP3), which releases Ca^{2+} from internal stores, and diacylglycerol (DAG), which activates protein kinase C (PKC). Activated G-protein cascades can interact to regulate adenylate cyclase function. Regulators of AC include Ca^{2+}, G protein subunit $\beta\gamma$, protein kinase A (PKA), PKC, and calmodulin-dependent kinase (CaMK) (Chern, 2000). Participants in the Gq cascade, like Ca^{2+}, CaMK, and PKC, can therefore regulate cascades mediated by Gs and Gi through adenylate cyclase. For example, stimulation of Gq-linked mGluR1 can enhance cAMP production (Aramori and Nakanishi, 1992) and stimulation of Gq-linked muscarinic and serotonin receptors potentiate AC6 and AC9 activity but the CaMK phosphorylation of AC1 and AC3 downstream of Gq is inhibitory to these AC enzymes (Beazely and Watts, 2005; Cumbay and Watts, 2005).

Cyclic AMP has emerged as a tightly regulated second messenger with multiple downstream effectors (Gilman, 1995). One known cAMP function is to serve as a second messenger that can activate cAMP-dependent PKA, an enzyme that phosphorylates tertiary messengers, which themselves can signal subsequent messengers. Alternatively cAMP can act directly as a transmitter by binding cyclic nucleotide gated channels (Matulef and Zagotta, 2003). Cyclic AMP produces short-term or long-term changes in neuron function by acting, respectively, through PKA or an EPAC-Rap-MAPK cascade involving an exchange protein activated by cAMP (EPAC), a small GTPase (Rap1), and a mitogen-activated protein kinase (MAPK) (Neves et al., 2002). Cyclic AMP subcellular distribution is not uniform (Rich et al., 2000) and its levels are tightly regulated by phosphodiesterases (PDEs). Regulation of cAMP levels is important because cAMP has several mechanisms to affect cellular function.

Phosphodiesterases provide the only means of degrading cAMP levels and maintaining cAMP homeostasis. Several isoforms of PDE are known to exist (Francis et al., 2001). PDE4 is a cAMP-specific PDE that is prevalent in the brain and is the target of antidepressant and cognitive enhancing pharmacotherapeutics. With activity being regulated by both cAMP and extracellular signal-regulated kinase (ERK) signaling, PDE4 is a key component in NMDA-mediated memory processes (Zhang et al., 2004). The PDE4 subfamilies PDE4A, PDE4B, PDE4C, and PDE4D are highly expressed in human brain regions, whereas rat brain primarily expresses PDE4A, B, and D (Cherry and Davis, 1999; Takahashi et al., 1999; Fujita et al., 2005; Parker et al., 2005). Cyclic AMP levels regulate the activity of PDE4 through cAMP-dependent PKA phosphorylation of PDE4 in the short term (Manganiello, 2002) and regulate the density of PDE4 through a positive feedback mechanism on PDE4 transcription in the long term (Beavo and Brunton, 2002; D'Sa et al., 2002; Conti et al., 2003; Houslay and Adams, 2003). Cyclic AMP itself can upregulate the expression of certain PDE4 variants (Conti and Jin, 1999; Houslay, 2001). PDE4 and cAMP levels are regulated through a feedback loop in which cAMP levels regulate PDE4 levels and PDE4 regulates cAMP levels.

3. Cyclic AMP in stress and anxiety

Mounting evidence from behavioral and pharmacological studies indicates that stress and anxiety are mediated through the cAMP second messenger system. While neural substrates of anxiety are known to involve the prefrontal cortex and the amygdala (Davidson, 2002), the importance of intracellular functional activity in these regions remains to be elucidated in humans. In rhesus monkeys, administration of diazepam, a benzodiazepine anxiolytic known to increase cAMP by inhibiting PDE cAMP hydrolysis (Cherry et al., 2001), produces increased activity in parietal areas and activation in frontal areas lateralized to the left (Davidson et al., 1992, 1993). Asymmetric distribution of activity reflects a role for diazepam-induced cAMP level increases in behavioral inhibition, increased positive affect, and decreased anxiety (Davidson et al., 1992, 1993). In the rat response to stress, intra and interhemispheric associations of intracellular functional activity in the hypothalamus, hippocampus, frontal cortex, and amygdala can be drawn from mapped increases of *in vivo* brain cAMP levels (Egorova et al., 2003). Rat pituitary increases cAMP production to multiple stressors (cold, forced running, formalin injection, immobilization, electric footshock) and the extent of cAMP increases corresponds to the intensity of the stressor (Kant et al., 1982). Based on microdialysis studies in rat, the brain contributes cAMP to plasma during stress (Stone and John, 1992). The cAMP cascade mediates stress and anxiety in localized brain

regions and the levels of cAMP are proportional to the intensity of the behavioral stressor.

The pharmacological mechanism of drug-induced anxiolysis can provide insight into the intracellular components contributing to behavioral anxiety. Several anxiolytic drugs exert their effect through the cAMP cascade. Diazepam, an anxiolytic benzodiazepine, and rolipram, a pyrrolidinone antidepressant with anxiolytic properties, mediate their anxiolytic effect through PDE inhibition, as do the methyl xanthines like caffeine. *In vitro* potency of several PDE inhibitors shows a positive correlation with the anxiolytic efficacy of rat performance on the Vomer conflict task (Beer et al., 1972). *In vivo* anxiolytic pharmacologic studies show a similar PDE inhibition mechanism. For example, diazepam inhibits mouse brain PDE4 subtypes transfected to human embryonic kidney cells (Cherry et al., 2001). Behavioral testing of the methyl xanthines, caffeine and pentoxifylline (a PDE4 inhibitor), confirms their effect as anxiolytics (Rao et al., 1999). During an elevated plus-maze test, the selective PDE4 inhibitor rolipram produces anxiolytic-like behavior in rats independent of simultaneous locomotor behavior (Silvestre et al., 1999). Thought to originate as intracellular cAMP, extracellular cAMP levels detected by microdialysis in rats increase in right medial prefrontal areas in response to a restraint stressor or peritoneal saline injection stressor (Stone and John, 1992). This effect was enhanced using rolipram, a selective inhibitor of PDE4 known to increase intracellular cAMP levels. The anxiolytic pharmacologic response appears to be mediated through a stress-reducing cAMP elevation. One possible pathway for increased cAMP levels to ultimately decrease anxiety is by directly upregulating expression of the cAMP response element (CRE) binding protein (CREB) (Nibuya et al., 1996). CREB is a transcription factor that binds CRE and increases the expression of neuropeptide Y (Higuchi et al., 1988), a peptide mediating anxiolysis (Pandey, 2003) found in corticolimbic structures like the amygdala (Millan, 2003). Pharmacologic studies indicate that the cAMP cascade is one mechanism that mediates anxiety and therapy that enhances the cAMP cascade in specific brain regions like the amygdala may be anxiolytic.

4. Cyclic AMP in memory

Deficiencies in the cAMP cascade result in memory deficits. This role has been elucidated by research in many systems including the gill withdrawal reflex of *Aplysia*; rutabaga, turnip, and dunce *Drosophila* mutants; and type 1a pseudohypoparathyroidism in humans (Berry-Kravis and Huttenlocher, 1992). In a mouse study, rolipram-induced increases of cAMP reduce the spatial memory defects associated with aging (Bach et al., 1999). The cAMP cascade is one of several implicated in studies of long-term potentiation (LTP), a neuronal model of memory (Lynch, 2004). LTP is considered a form of synaptic plasticity based on synaptic strengthening which is known to involve cAMP (Frey et al., 1993; Weisskopf et al., 1994), AMPA receptors, and NMDA receptors. For LTP, high frequency trains are required for glutamate to stimulate AMPA receptors to depolarize the post-synaptic cell and clear the magnesium block of NMDA channels to permit calcium flux. Insertion and recycling of AMPA receptors to the synapse requires cAMP-dependent PKA phosphorylation of the receptor (Esteban, 2003). In response to repetitive high frequency stimulation, NMDA receptors flux Ca^{2+} which can activate calcium-dependent AC1 and AC8 to increase cAMP production (Wong et al., 1999). LTP can be divided into an early, short-term phase and a late, long-term phase. The early phase is independent of cAMP and protein levels are unaltered whereas the late phase is cAMP dependent and is associated with protein synthesis (Ma et al., 1999). The involvement of the cAMP cascade in memory and LTP is well characterized in transgenics overexpressing AC1 (Wang et al., 2004) and AC1–AC8 double knockouts (Wong et al., 1999). Mice with a genetic increase in cAMP productivity due to AC1 overexpression have enhanced LTP with an associated increase in recognition memory behavior and AC1–AC8 double knockout mice, with a genetic reduction in cAMP productivity, do not produce late phase LTP and do not exhibit long-term memory. Pharmacologically targeting the cAMP system could enhance LTP and improve memory-related processes.

5. Fragile X memory and anxiety

5.1. Fragile X patients

Phenotypically, FX is characterized by mental retardation, cognition and memory deficits, autistic and stereotypic behaviors, developmental delays, hyperactivity, attention deficit disorder, and seizures (Reiss and Freund, 1990; Berry-Kravis, 2002; Hagerman, 2002). Memory deficits and anxiety-related social behavior are central to the FX full mutation phenotype (Cornish et al., 2001; Hagerman, 2002). For example, the FMRP defect impacts the anatomy (Reiss et al., 1994) and function of memory-related areas like the hippocampus. Reduced hippocampal and basal forebrain activation patterns are reported in a functional MRI (fMRI) study of FX patient-volunteers encoding a visual memory task (Greicius et al., 2004). In another fMRI study, lymphocytic FMRP levels are shown to have a direct relationship with regions of brain activated during a working memory task (Menon et al., 2000). Anxiety is commonly featured with memory deficits in FX individuals (Hagerman, 2002; Tsiouris and Brown, 2004). Although anxiety is difficult to detect in individuals with intellectual disability, behavioral equivalents of anxiety have been reported in FX (Sullivan et al., 2007) and are likely related to the hypersensitivity and hyperarousal (Cohen, 1995) described in electrodermal studies of FX individuals (Miller et al., 1999). As Hagerman notes (Hagerman, 2002), pharmacologic inhibition of the cAMP second messenger system prevents normal sensitization to sensory stimuli in *Aplysia* (Kandel and Schwartz, 1982) which has direct implications for the abnormal sensitization seen in FX individuals.

5.2. Fragile X fly

Drosophila melanogaster has an FMR1 homologue, *dfmr1*, in their genome. *Drosophila* express the *dfmr1* protein, *dfmrp*, in the central nervous system and specifically in mushroom bodies, key neural structures in learning and memory (Schenck et al., 2002). The *dfmr1* mutant, an FX fly model, has an altered mushroom body structure which contributes to the FX memory phenotype (Michel et al., 2004; Pan et al., 2004; Restifo, 2005). One example of an altered FX fly memory phenotype comes from assessment using conditioned courtship behavior, a model for memory in *Drosophila* (McBride et al., 1999). Briefly, a male fly will court a virgin female fly without training. Conditioned courtship suppression occurs when male flies suppress their courting behavior with virgin females after training in which the male, paired with a mated female, learns to reduce courtship activity. Although *dfmr1* mutants learn the paradigm when paired with mated females, the memory of training is absent when paired with virgin females. This memory defect in FX *Drosophila* behavior is improved with lithium and group II mGluR antagonists (LY341495, MPPG, MTPG), pharmaceuticals known to increase cAMP cascade function in flies (McBride et al., 2005). Based on the *dfmr1* mutant, impaired FX anxiety and memory behavior could be improved through cAMP pharmacotherapy.

5.3. Fragile X mouse

A knockout mouse model of FX syndrome was generated by insertion of a neomycin resistance cassette into exon 5 of the FMR1 gene (Bakker et al., 1994). The FMR1 knockout mouse exhibits deficits in neuronal pruning and alteration of FMRP-regulated protein synthesis (Comery et al., 1997; Nimchinsky et al., 2001). Since FMRP is thought to repress protein synthesis (Laggerbauer et al., 2001; Li et al., 2001), a loss of function mutation of the FMR1 allele is expected to enhance protein synthesis of FMRP-repressed proteins. Consistent with this view, radiotracer autoradiography indicates enhanced protein expression in hypothalamus, thalamus, basolateral amygdala, hippocampus, frontal association, and posterior parietal cortex of FMR1 knockout mice (Qin et al., 2005).

Targeted disruption of FMR1 results in several mouse phenotypes that share commonalities with human FX and autism behavior, but there are also some potential differences (for further review, see Bernardet and Crusio, 2006). Depending upon genetic background, behavioral and synaptic models of memory in mice are consistent with the human FX phenotype (for background, see Lombroso, 2003; Hessl et al., 2004). Measures of learning and memory in a fear based paradigm were generally comparable for targeted and wild-type alleles on a C57BL/6 background (Peier et al., 2000), though these mice expressed reduced memory for avoidance in a leverpress paradigm compared to wild type (Brennan et al., 2006). Lack of functional FMR1 alleles on an FVB/NJ background resulted in deficits in a passive avoidance memory task (Qin et al., 2002). The memory defect in gene-targeted mice C57BL/6J is not due to an FMRP-reduction of CREB, since CREB levels are comparable for targeted and wild-type alleles (Li et al., 2002). However, if cAMP levels are reduced in FX, then CREB activity might be attenuated, resulting in downstream memory deficits. Since LTP is a synaptic model of memory dependent upon cAMP expression, FMR1 knockout mice were also evaluated for late-phase LTP in the hippocampus. No differences in LTP were identified between targeted and wild-type alleles in hippocampus for mice with the C57BL/6 (Paradee et al., 1999) or C57BL/6J (Li et al., 2002) background. However, FMR1 knockouts (C57BL/6J) expressed reduced LTP in cortex relative to wild type, possibly due in part to significantly reduced cortical expression of the AMPA receptor GluR1 subunit (Li et al., 2002). A recent patch clamp study of trace fear memory in the FMR1 knockout mouse (FVB.129P2-*Fmr1*^{tm1CgR}) found reduced LTP in anterior cingulate cortex and lateral amygdala (Zhao et al., 2005). Taken together, the differences in memory behavior and LTP among studies of knockout strains reflect characteristics of the experimental paradigm, brain region dependence, and could also reflect alterations in cAMP cascade function across brain regions.

Anxiety is commonly featured in human FX and has been extensively reviewed (Hagerman, 2002; Tsiouris and Brown, 2004). However, behavioral and physiological studies of mouse anxiety suggest abnormal, but not typically heightened anxiety responses in FMR1 knockout mice (for thorough review, see Bernardet and Crusio, 2006). For example, in the FVB/NJ strain, knockout mice show reduced anxiety-related behavior in an open field test compared to wild type (Qin et al., 2002). FMR1 knockouts on a C57BL/6 background, however, express heightened open field exploration and light–dark exploration relative to wild-type controls (Peier et al., 2000). In the C57BL/6 background, anxiety-related behaviors associated with non-functional FMR1 alleles can be rescued via introduction of a yeast artificial chromosome (YAC) bearing a functional copy of the human FMR1 gene (Peier et al., 2000).

While several behavioral phenotypes of the FMR1 knockout mouse are consistent with human autism (Bernardet and Crusio, 2006), the abnormal anxiety phenotype suggests that the FX model does not completely model the human condition. Several explanations could account for a possible discrepancy, which we would like to address since it is critical to our model for cAMP's role in the modulation of FX anxiety. One possible explanation is that the existing mouse behavioral tests for anxiety do not represent features of human anxiety. The open field test is commonly employed to measure anxiety-like behaviors but several mechanisms could motivate a rodent to withdraw from an open field environment. For example, open exposure to a light source might feel aversive to a mouse without provoking anxiety. Alternatively, areas adjacent to walls might feel more rewarding. Outstanding empirical and theoretical treatments on the influence of subjective experience on seeking and avoidance behaviors is provided by Theodore Schneirla (Maier and Schneirla, 1942; Schneirla, 1959). Importantly, mouse anxiety is only one of many subjective states that explains open field avoidance. Rigorous theoretical and experimental models of the underlying neurobiology of subjective experience are required to gain a critical comparative understanding of affective phenotypes (Panksepp, 1998). More novel work with mirrored chambers (Spencer et al., 2005) may provide greater insight into the motivations that modulate mouse behavioral phenotypes.

Another possible explanation is that cyclic AMP does not modulate anxiety phenotypes in FMR1 knockout mice in the same fashion that anxiety is modulated in humans. Perhaps we can gain insight into FMR1 knockout anxiety from studies of mouse social behavior. Anxiety has been shown to diminish social interaction (File and Hyde, 1978; File and Seth, 2003). Some studies suggest that social behaviors of FX-targeted alleles can be similar to or even enhanced relative to wild-type alleles, depending upon the specifics of test design (Spencer et al., 2005; Brennan et al., 2006), indicating again that lack of a functional FMR1 allele does not enhance social anxiety. However, more recent work, that employed behavioral measures of social amnesia (Ferguson et al., 2000), finds diminished social approach responses of the FMR1-targeted mice (Mineur et al., 2006), suggesting the possibility of deficits within the social domain. While levels of social approach can be inversely associated with anxiety levels, they can also be associated with differences in social reward (Panksepp and Lahvis, 2007). Since the neural pathways for the subjective experiences of reward and fear/anxiety are respectively supported, in part, by striatal nuclei, such as the nucleus accumbens (Panksepp et al., 1994; Kelley et al., 2005), and the amygdala (Rodrigues et al., 2004), then primarily cortical alterations to the cAMP system in FMR1 knockout mouse (Fig. 1) would have a lesser impact on the subcortical mediation of mouse social or anxiety behaviors.

As a final explanation, the gene targeting and genetic background of the knockout mouse may preserve some aspects of the phenotype, but not all of them. A neomycin resistance cassette is in the FMR1 gene construct (Bakker et al., 1994) and its location and orientation can modulate the function of neighboring genes. Orientation of the neomycin resistance cassette, for example, can modify phenotypes through *cis* effects (Lahvis and Bradfield, 1998). Genetic background can also modulate phenotypes. Because FMR1-targeted mice are primarily evaluated on only two backgrounds (C57BL/6 and FVB), more representative models of human FX should be generated on other genetic backgrounds. Other gene-targeting strategies and use of different genetic backgrounds might generate anxiety phenotypes without discrepancies from the human FX phenotype.

In this sense, more recent gene-targeting strategies provide exciting new mouse models of fragile X syndrome. Knock-in mice with an expansion of CGG repeats in the FMR1 promoter have been developed to understand the human FX premutation and its

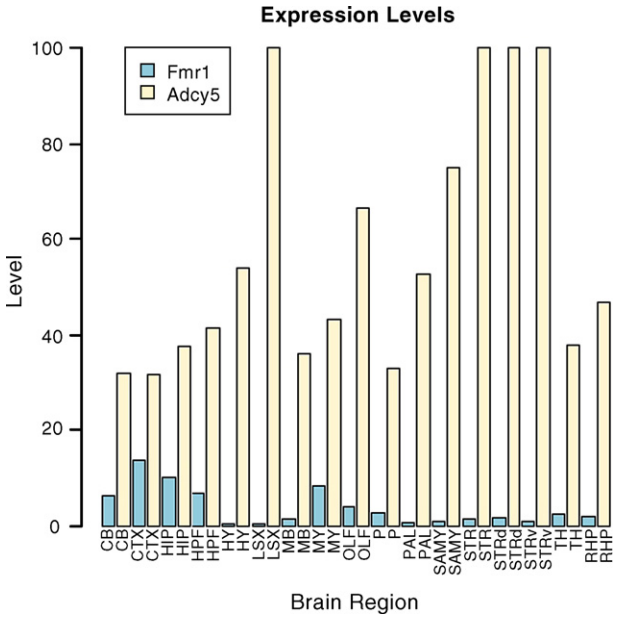

Fig. 1. Expression summary of FMR1 and adenylate cyclase type V. In normal mice, cortical expression of FMR1 is greatest in cortex but adenylate cyclase expression is ubiquitous. Data available from (http://www.brain-map.org). CB, cerebellum; CTX, cerebral cortex; HIP, hippocampal region; HPF, hippocampal formation; HY, hypothalamus; LSX, lateral septal complex; MB, midbrain; MY, medulla; OLF, olfactory areas; P, pons; PAL, pallidum; sAMY, striatum-like amygdalar nuclei; STR, striatum; STRd, striatum dorsal region; STRv, striatum ventral region; TH, thalamus; RHP, retrohippocampal region.

expansion. Mice engineered using a yeast artificial chromosome (YAC) with a $(CGG)_{98}$ human premutation (Bontekoe et al., 2001; Willemsen et al., 2003) have several features in common with the human FX premutation tremor-ataxia phenotype (Greco et al., 2002). An FX knock-in premutation mouse engineered using serial ligation (SL) methodology (Grabczyk and Usdin, 1999), rather than with a YAC, has reduced FMRP expression that is associated with repeat length and varies in its extent across the brain. Unlike the YAC knock-in mouse (Brouwer et al., 2007), the SL knock-in mouse can also expand its repeat length to a full mutation in one generation (Entezam et al., 2007). Premutation knock-in mice could prove useful to model the cognitive decline of memory in the FX premutation and full mutation phenotype. To assess the value of using FX targeting to generate useful mouse models of autistic phenotypes, it would be very interesting to examine the social and anxiety-related behaviors in these mouse models.

6. Fragile X signaling theories

6.1. mGluR theory of fragile X

Signaling alterations are central to FX pathophysiology (Miyashiro and Eberwine, 2004). Using the FX mouse model, mGluR1 and mGluR5 mediated long-term depression (LTD), a model for the weakening of synapses, was found to be enhanced and has been formulated into the mGluR theory of FX (Bear et al., 2004). In this theory, overstimulation of mGluR1 and mGlur5 leads to enhanced synthesis of proteins normally repressed by FMRP through enhanced PKC activity. Mechanistically, the binding of glutamate to mGluR1 and mGluR5 receptors activates the Gq cascade in which phospholipase C cleaves phosphatidylinositol bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 releases calcium from internal stores and DAG activates PKC to increase production of proteins normally repressed by FMRP. This FX theory is not specific to the mGluR1 and mGluR5 receptors but is likely generalizable to a PKC-mediated defect (Weiler et al., 2004) or to other receptors signaling through Gq (Volk et al., 2007). Due to mGluR1 and mGluR5 overstimulation, AMPA and NMDA receptors are thought to be withdrawn from the neuronal membrane producing weakening of the synapse and LTD. The basis for FX treatment with AMPAkines is to enhance the open duration of the remaining AMPA receptors to promote strengthening at the synapse (Berry-Kravis and Potanos, 2004). Results from a recent clinical trial of AMPAkines were inconclusive based on low dosing (Berry-Kravis et al., 2006). Pharmacologic rescue of FX *Drosophila* behavior by an mGluR antagonist supports the mGluR theory (McBride et al., 2005). The consequences of FMRP loss on signaling through mGluR1 and mGluR5 receptors, and more generally through the Gq cascade, contributes to the FX phenotype.

6.2. Cyclic AMP theory of fragile X

Berry-Kravis et al. argue that altered FX cAMP metabolism contributes to the FX neurobehavioral phenotype based on studies of several non-neural cell types which showed that the alteration in cAMP induction is FMRP dependent and occurs at the level of adenylate cyclase or its regulation (Berry-Kravis, 1990; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998). We have termed this line of thought the cAMP theory of FX (Kelley et al., 2006, 2007) to distinguish it from the mGluR theory (Bear et al., 2004).

Decreased cAMP levels are noted in human FX through investigation of various cell types used to model intracellular neuronal activity. In platelets, induced cAMP production is significantly lower in patients with FX relative to non-FX controls (Berry-Kravis and Huttenlocher, 1992) and also relative to patients with mental retardation, autism, and normal intelligent controls (Berry-Kravis and Sklena, 1993). Although not statistically significant, basal platelet cAMP production tended to be reduced in FX to about 72% that of controls (Berry-Kravis, 1992). In lymphoblastoid cell lines of FX patients, a significant negative correlation exists between levels of induced nonreceptor-mediated cAMP production and trinucleotide repeat amplification (Berry-Kravis et al., 1995). Overexpression of FMR1 in HN2 mouse neural cells increases levels of cAMP and shows a positive correlation between FMRP and cAMP levels (Berry-Kravis and Ciurlionis, 1998). These results suggest that FMRP regulates cAMP levels and, in FX, reduced FMRP levels correspond to reduced stimulated cAMP levels (Berry-Kravis and Ciurlionis, 1998). While the experimental manipulations of cAMP levels and the over-expression of FMRP in a cell culture system likely fall outside the range of normal cell physiology, the results from these studies point to an alteration in cAMP cascade function that is FMRP dependent. In support of these cellular studies, FX *Drosophila* behavior is improved with lithium and a group II mGluR antagonist, a pharmaceutical known to increase cAMP cascade function (McBride et al., 2005). Therefore, a relationship between cAMP, FMRP, and FX behavior was established in non-neural cells and FX fly but remained to be tested in brain tissue.

Cyclic AMP production in the FX brain was examined for the first time (Kelley et al., 2007) by comparing cAMP levels across fly heads, mouse cortex, and human neural cell lines (Bhattacharyya et al., 2008). Induced cAMP levels are reduced in FX and all three models have deficient stimulated cAMP production. This study suggests that neurotransmitters and receptors signaling through cAMP would be functionally deficient in FX as would the influence of cAMP on cAMP-gated channels, GEFs, and EPAC signaling (Lauder, 1993; Neves et al., 2002; Matulef and Zagotta, 2003). If

cAMP induction is reduced across neural cell types, all members of the tripartite synapse (Araque et al., 1999) could be compromised with respect to cAMP signaling. In neurons, the consequences of this FX cAMP induction impairment must be considered not only postsynaptically, but also presynaptically. Cyclic AMP has a known role in neurotransmitter release from the presynaptic terminal (Kaneko and Takahashi, 2004). In FX, reduced cAMP induction at the presynaptic terminal would presumably reduce the inhibition of group II and III mGluR and, depending on splice variant, the group 1 family which would result in a reduced inhibition of glutamate release from the presynaptic terminal (Serajee et al., 2003). Taken together, the cAMP cascade is an important signaling pathway deficient in FX and further studies are necessary to characterize cAMP deficiencies at multiple loci of the tripartite synapse.

According to the cAMP theory of fragile X, a loss of FMRP produces cAMP-dependent alterations in functional neurophysiology. Cyclic AMP signal transduction, which is involved in neurotransmission (Florendo et al., 1971), neuroplasticity, and mnemonic processes (MacKenzie et al., 2002), is a molecular mechanism (Walton and Rehfuss, 1990) by which FMRP can affect protein expression (Kaufmann and Reiss, 1999). If altered, the production of intracellular cAMP would be disproportional to that demanded by hormones or extracellular neurotransmitters, like epinephrine, dopamine, and serotonin (Lauder, 1993). The consequence of cAMP dysregulation in FX would produce a loss of a neurotransmitter's ability to control postsynaptic neuronal cAMP activity. This, in turn, ultimately produces dysfunctionally connected neural circuits with behavioral consequences.

Lower levels of PDE4 are likely required in FX to maintain cAMP homeostasis because PDE4 levels are regulated by cAMP levels with expression of some PDE4 subgroups potentially being mediated through a cAMP-dependent CREB–promoter interaction (Houslay, 2001). The levels of cAMP and PDE4 are prone to vary with FMRP across the brain because the level by which FMRP expression is reduced can vary among FX brain regions (Taylor et al., 1999) and there is a direct correlation between levels of cAMP and FMRP (Berry-Kravis and Ciurlionis, 1998). The extent to which cAMP and PDE4 levels vary with FMRP levels across brain regions could be studied with C^{11}-rolipram using PET (DaSilva et al., 2002). The cAMP defect would be expected to occur more in the cortex compared to other brain regions based on data from the Allen Brain Atlas (Lein et al., 2007) which shows that FMR1 is primarily colocalized with AC type 5 in cortex (Fig. 1).

The FX neurophenotype has distinctive features which are explained in part by cAMP alterations during neural differentiation. The developmental responsiveness to adenylate cyclase stimulation may be a useful biochemical marker to monitor neural maturation (Seed and Gilman, 1971). The cAMP cascade is known to be developmentally regulated in mouse and rat brain (Schmidt et al., 1970; Rius et al., 1991, 1994). Studies examining development of the cAMP cascade in normal mice indicate that the inhibitory influence on adenylate cyclase (AC) early in development is followed by stimulatory AC responsiveness later in development (Rius et al., 1991, 1994). Developmentally, dysregulation of the cAMP cascade is known to impact growth cone motility and the structure of terminal varicosities in *Drosophila* (Kim and Wu, 1996). Cyclic AMP plays an important role in AMPA trafficking (Lu et al., 2003), cell survival and neurogenesis (Nakagawa et al., 2002), neural induction (Otte et al., 1989), neurite formation, and synaptogenesis (Takuro Tojima and Etsuro Ito, 2003). Cyclic AMP also regulates spine density by gating brain derived neurotrophic factor (BDNF) signaling, a neurotrophin implicated in dendrite formation (Ji et al., 2005). Several of these features are altered in FX. In addition, EphB2, a key component in dendritic spine formation with contributions to abnormal pruning in FX (Ethell et al., 2001; Henkemeyer and Frisen, 2001; Irie and Yamaguchi, 2004), is a likely candidate to influence development of cAMP responsiveness to forskolin (Ibrisimovic et al., 2007). The mechanism for the distinctive development of the FX cAMP response remains unknown (Kelley et al., 2007) but the maturation of AC responsiveness in FX is likely related to FMRP loss or its downstream consequences.

In the context of the mGluR theory (Bear et al., 2004), it is of interest to note that Ca^{2+} and PKC, both of which are part of the Gq cascade, are able to regulate certain AC isozymes and impair their functional ability. For instance, phosphorylation of AC2 and AC4 by PKC weakens hippocampal synaptic plasticity by reducing the ability of these ACs to integrate signal from Gsα and Giα receptors (Chern, 2000). The mGluR1 and mGluR5 oversignaling through PKC proposed in the FX mGluR theory enhances the activity of certain AC isozymes, such as type 1 ACs through Gq-related calcium increases, to increase cAMP counter to the cAMP theory of FX (Chern, 2000). Clearly there is evidence that cyclic AMP and mGluR interact because a given G protein has the potential to act through a network of multiple overlapping messengers (Neves et al., 2002). Several lines of evidence support the view that mGluR and cAMP-mediated pathways interact in platelets (Moos and Goldberg, 1988), lymphocytes (Pacheco et al., 2004), neurons (Otte et al., 1989; Pilc et al., 1996; Cartmell et al., 1997; Azad et al., 2004; Warwick et al., 2005) and astrocytes (Balazs et al., 1998). This means that the impaired cAMP signaling in the cAMP theory of FX could be related to the Gq signaling described in the mGluR theory. The group I mGluR family can interact with the cAMP cascade through a G protein mechanism (Thomsen, 1996; Balazs et al., 1998) involving Gs and Gq (Tateyama and Kubo, 2006). The coupling of the mGluR1 receptor to a given cascade has been localized to the carboxy terminal (Gabellini et al., 1993) and to intracellular loops two and three (Francesconi and Duvoisin, 1998). Further studies are necessary to determine whether the cAMP and mGluR mechanisms occur in series or in parallel (Kelley et al., 2007).

7. Cyclic AMP in autism

The high comorbidity of autism and FX (Brown et al., 1982b, 1986; Loesch et al., 2007) warrants a discussion of cAMP function in autism. To date, no studies of the neuronal cAMP cascade are available in autism or autism comorbid with FX. However, cAMP levels in cerebrospinal fluid (CSF) and peripheral blood levels of cAMP in platelets and plasma are documented in autism. In autism, probenicid-induced increases in CSF cAMP levels in children are not different from previously reported baseline levels in adults (Winsberg et al., 1980). Autism and controls had comparable levels of stimulated cAMP production in platelets (Berry-Kravis, 1992; Berry-Kravis and Sklena, 1993), potentially useful models of neural signal transduction (Stahl, 1977; Camacho and Dimsdale, 2000). Plasma cAMP levels have been used as a physiologic indicator of sympathetic arousal (Huhman et al., 1991), emotional stress (Arnetz et al., 1979), noise related stress-induced anxiety (Iwamoto et al., 1995), childhood psychoses (Goldberg, 1984), and hyperkinetic behavior of autism and mental retardation (Hoshino et al., 1980). Plasma levels of cAMP are elevated in medicated and unmedicated autistic children relative to controls, but plasma cAMP levels are reduced relative to mentally retarded patients with hyperkinetic disorder (Hoshino et al., 1980; Cook, 1990). Increased plasma cyclic AMP levels in autism are significantly and positively associated with hyperkinesia and serum serotonin levels (Hoshino et al., 1979). However, since a variety of tissues contribute to plasma cAMP levels, the inter-

pretation of plasma cAMP studies is not specific to brain (Goldberg, 1984) and could be explained by alternative pathways such as hepatorenal plasma cAMP signaling (Ahloulay et al., 1996). Although CSF and peripheral blood measures indicate that cAMP levels are normal to high in autism, the cAMP cascade remains to be studied in the autistic brain.

Pharmacological studies based on theoretical models of autism provide further evidence for the role of cAMP pathology in autism. There are several agents known to affect cAMP that are therapeutic in autism. To account for the altered social and emotional behavior in autism, the opiate theory of autism proposes that autists have a hyperactive opiate system (Panksepp, 1979). Opioids are known to reduce the ability of adenylate cyclase to produce cAMP (Koski and Klee, 1981; Childers et al., 1986; Childers, 1988; Prather et al., 1994). Several autism studies indicate that naltrexone, an opiod antagonist which would improve adenylate cyclase function, is beneficial to some aspects of autism behavior in some patients (Kolmen et al., 1995; Riddle et al., 1999; Elchaar et al., 2006) but not all behaviors (Zingarelli et al., 1992; Willemsen-Swinkels et al., 1995; Feldman et al., 1999). The fetal valproate model of autism was based on case reports of the increased incidence of autism in individuals with fetal exposure to the maternal anti-epileptic valproate (Williams et al., 2001). Valproate has several mechanisms of action and is known to reduce stimulated, but not basal, cAMP levels (Montezinho et al., 2007). Mice exposed to valproate during development exhibit functional deficits that parallel autism (Ingram et al., 2000; Wagner et al., 2006). The impact of valproate on the functional overconnectivity of cortical microcircuitry (Rinaldi et al., 2007) has implications for the system level functional connectivity alterations noted in autism. Secretin is a neuropeptide that increases intracellular cAMP levels by activating adenylate cyclase upon binding its type II Gs coupled receptor, which has been linked to social behavior in mice (Nishijima et al., 2006; Siu et al., 2006). A case study (Horvath et al., 1998) reported improvement in autism behavior with infusion of secretin, although further clinical trials do not support the efficacy of this approach (Esch and Carr, 2004; Sturmey, 2005; Williams et al., 2005). Based on these theories and the studies testing them, elevated cyclic AMP in CSF and peripheral blood may reflect a compensatory mechanism to increase low cAMP levels.

Screens of genes associated with susceptibility to autism have also implicated cAMP signaling in the autism phenotype. Variants in the cAMP guanine nucleotide exchange factor II (cAMP-GEFII) gene are associated with the autism phenotype (Bacchelli et al., 2003). cAMP-GEFII codes for a regulator of G proteins that is functionally dependent on cAMP levels and is involved in neuronal development and exocytotic processes. The mGluR8 receptor has an inhibitory effect on cAMP signaling; and, variants in the mGluR8 gene, GRM8, at 7q31 are associated with an increased susceptibility to autism (Serajee et al., 2003). The cAMP cascade remains to be studied directly in autism brain and has potential utility as a target to enhance memory for behavioral therapies.

8. Cyclic AMP therapy

Treatment through the cAMP cascade may be beneficial in FX. Cyclic AMP was thought to regulate FMRP expression through an FMR1 enhancer (Hwu et al., 1997). However, a recent PC12 study showed that FMR1 is not cAMP inducible, presumably due in part to the absence of a TATA box in the FMR1 promoter region, even though FMR1 is bound by phospho-CREB/ATF (Smith et al., 2006). Nevertheless, therapies working through cyclic AMP transduction could be beneficial through indirect effects on FX neurophysiology. In particular, FX memory deficits (Greicius et al., 2004) and hypersensitivity to stress (Barad et al., 1998) are candidate behaviors that can be improved by enhancing cAMP levels.

With pharmaceutical calibration of FX cAMP levels, the intracellular cAMP signaling cascade would be able to properly regulate the cAMP second messenger in response to extracellular signals (Chen et al., 1999) to improve FX behavior. For example, the mood stabilizer lithium increases cAMP production (Chen et al., 1999) and is known to improve FX *Drosophila* memory behavior, as have group II mGluR antagonists which elevate cAMP levels (McBride et al., 2005). Enhancing cAMP production strengthens glutamatergic synapses by augmenting the function of AMPA receptors (Chavez-Noriega and Stevens, 1992; Tseng and O'Donnell, 2004). AMPA receptors are reduced in FX and are pharmaceutical targets of interest (Bear et al., 2004; Berry-Kravis and Potanos, 2004). Among pharmaceuticals, forskolin (Seamon and Daly, 1986) is a diterpine extracted from the root of Coleus forskohlii, a member of the mint family, that is a reversible activator of adenylate cyclase (AC) in a number of cells including neurons (Seamon et al., 1981). Forskolin is a useful tool in characterizing AC function and the AC response to extracellular or intracellular modulators (Insel and Ostrom, 2003). In a step-down active avoidance task, long-term memory in rats improved when forskolin was directly infused into hippocampus (Bernabeu et al., 1997). As an over-the-counter herbal, forskolin is a nootropic with the potential to enhance cAMP-mediated memory processes in FX. Rolipram, 4-(3-cyclpentyloxy-4-methoxyphenyl)-2-pyrrolidinone, is a potent PDE4 inhibitor, a class that shows clinical potential in the treatment of depression. Through cAMP-mediated mechanisms, pharmacotherapy that inhibits PDE4 is prone to increase the transcription and function (Miller et al., 2002; Pace et al., 2007) of reduced numbers of FX dendritic glucocorticoid receptors (Miyashiro et al., 2003) involved in hypothalamic-pituitary–adrenal (HPA) regulation (Hessl et al., 2004). A clinical trial of rolipram reported acceptable tolerance levels at clinically relevant doses (Fleischhacker et al., 1992). In clinical and preclinical trials, the main human PDE4 inhibitor side effect, thought to be due to PDE4D activity (Robichaud et al., 2002a,b; Conti et al., 2003), is emesis at clinical doses while rats are more susceptible to toxic effects like arteriopathy. One explanation for this effect is the reduced PDE4 enzyme activity in humans relative to monkeys and rats (Bian et al., 2004).

The prospect of using PDE4 inhibitors like rolipram is particularly appealing because memory enhancement through facilitation of cAMP signaling is possible without changes in basal cAMP levels (Barad et al., 1998). Several new PDE4 inhibitors are or are likely to become available and to provide new avenues of therapy for patients with FX. HT0712 (Inflazyme PDE4 Library IPL455, 903) is a PDE4 inhibitor produced by Helicon Therapeutics that is currently in Phase 1 human clinical trials. In a mouse model of Rubinstein-Taybi mental retardation, both HT0712 and rolipram rescued object recognition memory processes which were deficient relative to wild-type (Bourtchouladze et al., 2003). MEM1018 and MEM1091 are PDE4 inhibitors that behave much like rolipram with comparable PDE4 inhibitory activity profiles. MEM1018, MEM1091, and rolipram can restore pharmacologically induced impairment of long-term memory and potentiate NMDA-stimulated cAMP production (Zhang et al., 2005). Based on the cAMP theory, pharmacotherapies modulating the cAMP cascade have potential as both a nootropic to improve mnemonic processing and an anxiolytic.

9. Conclusion

The cAMP system is known to be involved in mnemonic processing and anxiogenesis. Defects in the cAMP cascade

potentially contribute to the phenotypic features of these behaviors in FX and autism. Cyclic AMP signaling is reduced in FX but may be normal or enhanced in autism brain. Cyclic AMP is seated to be a useful biochemical marker in distinguishing autism per se from the autism noted in patients with FX. In patients with FX comorbid with autism, reduced cAMP signaling would suggest that the autism noted in FX is unique whereas a normal to high cAMP level would indicate that autism may be a compensatory process to increase cAMP levels. Further studies are needed in brain to test this hypothesis and to determine the efficacy of treatment through the cAMP cascade. Treatment approaches through pharmacologic manipulation of the cAMP cascade have the potential to benefit FX and autism individuals by alleviating anxiety and improving mnemonic processing.

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