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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.

Keywords

Autism; Fragile X; Cyclic AMP; cAMP; signal transduction; FMRP; FMR1

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 retardation-1 (FMR1) gene that suppresses production of fragile X mental retardation protein (FMRP) (Pieretti, Zhang et al. 1991; Verkerk, Pieretti et al. 1991; Kaufmann, Cohen

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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, Kuhl et al. 1991). The comorbidity of autism and FX was initially reported to be around 18% (Brown, Jenkins et al. 1982) and a lower percentage of those with autism have FX (about 7%) (Brown, Friedman et al. 1982; Brown, Jenkins et al. 1986; Loesch, Bui 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, Goldstein et al. 2006; Gillott and Standen 2007) and FX behavioral phenotypes (Cornish, Munir 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, Hicar 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.

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 ten 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 opioid 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, Vanhauwe et al. 2003). Gs activates all membrane bound AC isoforms and forskolin, an AC activator (Seamon, Padgett 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 biphosphate (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 down stream 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, Ram et al. 2002). Cyclic AMP subcellular distribution is not uniform (Rich, Fagan 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, Turko 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, Zhao 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, Terwilliger et al. 1999; Fujita, Zoghbi et al. 2005; Parker, Matthews 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, Tolbert et al. 2002; Conti, Richter 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.

cAMP 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, Thompson et al. 2001), produces increased activity in parietal areas and activation in frontal areas lateralized to the left (Davidson, Kalin et al. 1992; Davidson, Kalin et al. 1993). Asymmetric distribution of activity reflects a role for diazepam-induced cAMP level increases in behavioral inhibition, increased positive affect, and decreased anxiety (Davidson, Kalin et al. 1992; Davidson, Kalin et al. 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, Stepanichev 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, Meyerhoff 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 show a positive correlation with the anxiolytic efficacy of rat performance on the Vomer conflict task (Beer, Chasin 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, Thompson et al. 2001).

Behavioral testing of the methyl xanthines, caffeine and pentoxifylline (a PDE4 inhibitor), confirms their effect as anxiolytics (Rao, Santos 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, Fernandez 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, Nestler et al. 1996). CREB is a transcription factor that binds CRE and increases the expression of neuropeptide Y (Higuchi, Yang 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.

cAMP 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, Barad 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, Huang et al. 1993; Weisskopf, Castillo 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, Athos 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, Zablow et al. 1999). The involvement of the cAMP cascade in memory and LTP is well characterized in transgenics overexpressing AC1 (Wang, Ferguson et al. 2004) and AC1-AC8 double knockouts (Wong, Athos 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.

FX Memory and Anxiety

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, Munir et al. 2001; Hagerman 2002). For example, the FMRP defect impacts the anatomy (Reiss, Lee 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, Boyett-Anderson 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, Kwon 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, Hooper et al. 2007) and are likely related to the hypersensitivity and hyperarousal (Cohen 1995) described in electrodermal studies of FX individuals (Miller, McIntosh 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.

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, Van de Bor et al. 2002). The *dfmr1* mutant, an FX fly model, has an altered mushroom body structure which contributes to the FX memory phenotype (Michel, Kraft et al. 2004; Pan, Zhang 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, Giuliani 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, Choi et al. 2005). Based on the *dfmr1* mutant, impaired FX anxiety and memory behavior could be improved through cAMP pharmacotherapy.

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, Verheij et al. 1994). The FMR1 knockout mouse exhibits deficits in neuronal pruning and alteration of FMRP-regulated protein synthesis (Comery, Harris et al. 1997; Nimchinsky, Oberlander et al. 2001). Since FMRP is thought to repress protein synthesis (Laggerbauer, Ostareck et al. 2001; Li, Zhang 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, Kang 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; Hessler, Rivera 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, McIlwain et al. 2000), though these mice expressed reduced memory for avoidance in a leverpress paradigm compared to wild type (Brennan, Albeck et al. 2006). Lack of functional FMR1 alleles on an FVB/NJ background resulted in deficits in a

passive avoidance memory task (Qin, Kang 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, Pelletier 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, Melikian et al. 1999) or C57BL/6J (Li, Pelletier 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, Pelletier 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, Toyoda 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, Kang 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, McIlwain 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, McIlwain 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, Alekseyenko 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, Alekseyenko

et al. 2005; Brennan, Albeck 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, Young et al. 2000), finds diminished social approach responses of the FMR1-targeted mice (Mineur, Huynh 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, Normansell et al. 1994; Kelley, Schiltz et al. 2005), and the amygdala (Rodrigues, Schafe et al. 2004), then primarily cortical alterations to the cAMP system in FMR1 knockout mouse (Figure 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, Verheij 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 expansion. Mice engineered using a yeast artificial chromosome (YAC) with a (CGG)₉₈ human premutation (Bontekoe, Bakker et al. 2001; Willemsen, Hoogeveen-Westerveld et al. 2003) have several features in common with the human FX premutation tremor-ataxia phenotype (Greco, Hagerman 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, Mientjes et al. 2007), the SL knock-in mouse can also expand its repeat length to a full mutation in one generation (Entezam, Biacsi 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.

Fragile X Signaling Theories

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, Huber 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, Spangler et al. 2004) or to other receptors signaling through Gq (Volk, Pfeiffer 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 AMPAkinases 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 AMPAkinases were inconclusive based on low dosing (Berry-Kravis, Krause et al. 2006). Pharmacologic rescue of FX *Drosophila* behavior by an mGluR antagonist supports the mGluR theory (McBride, Choi 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.

Cyclic AMP Theory of Fragile X

Berry-Kravis and colleagues 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, Hicar et al. 1995; Berry-Kravis and Ciurlionis 1998). We have termed this line of thought the cAMP theory of FX (Kelley, Davidson et al. 2006; Kelley, Davidson et al. 2007) to distinguish it from the mGluR theory (Bear, Huber 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, Hicar 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 overexpression 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, Choi 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, Davidson et al. 2007) by comparing cAMP levels across fly heads, mouse cortex, and human neural cell lines (Bhattacharyya, McMillan 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, Ram et al. 2002; Matulef and Zagotta 2003). If cAMP induction is reduced across neural cell types, all members of the tripartite synapse (Araque, Parpura 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 I family which would result in a reduced inhibition of glutamate release from the presynaptic terminal (Serajee, Zhong 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, Barnett et al. 1971), neuroplasticity, and mnemonic processes (MacKenzie, Baillie 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, Tassone 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 C¹¹-rolipram using PET (DaSilva, Lourenco 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, Hawrylycz et al. 2007) which shows that FMR1 is primarily colocalized with AC type 5 in cortex (Figure 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, Palmer et al. 1970; Rius, Streaty et al. 1991; Rius, Mollner et al. 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, Streaty et al. 1991; Rius, Mollner et al. 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, She et al. 2003), cell survival and neurogenesis (Nakagawa, Kim et al. 2002), neural induction (Otte, van Run et al. 1989), neurite formation, and synaptogenesis (Takuro Tojima 2003). Cyclic AMP also regulates spine density by gating brain derived neurotrophic factor (BDNF) signaling, a neurotrophin implicated in dendrite formation (Ji, Pang 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, Irie et al. 2001; Henkemeyer and Frisen 2001; Irie and Yamaguchi 2004), is a likely candidate to influence development of cAMP responsiveness to forskolin (Ibrisimovic, Bilban et al. 2007). The mechanism for the distinctive development of the FX cAMP response remains unknown (Kelley, Davidson 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, Huber 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 $\text{Gs}\alpha$ and $\text{Gi}\alpha$ 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, Ram 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, Ciruela et al. 2004), neurons (Otte, van Run et al. 1989; Pilc, Legutko et al. 1996; Cartmell, Schaffhauser et al. 1997; Azad, Monory et al. 2004; Warwick, Nahorski et al. 2005) and astrocytes (Balazs, Miller 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, Miller 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, Manev 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, Davidson et al. 2007).

cAMP in Autism

The high comorbidity of autism and FX (Brown, Jenkins et al. 1982; Brown, Jenkins et al. 1986; Loesch, Bui 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, probenecid induced increases in CSF cAMP levels in children are not different from previously reported baseline levels in adults (Winsberg, Sverd 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, Hebert et al. 1991), emotional stress (Arnetz 1979), noise related stress-induced anxiety (Iwamoto, Ishii et al. 1995), childhood psychoses (Goldberg 1984), and hyperkinetic behavior of autism and mental retardation (Hoshino, Kumashiro 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, Kumashiro et al. 1980; Cook 1990). Increased plasma cyclic AMP levels in autism are significantly and positively associated with hyperkinesia and serum serotonin levels (Hoshino, Kumashiro et al. 1979). However, since a variety of tissues contribute to plasma cAMP levels, the interpretation 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, Dechaux 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, Nijssen et al. 1986; Childers 1988; Prather, Tsai et al. 1994). Several autism studies indicate that naltrexone, an opioid antagonist which would improve adenylate cyclase function, is beneficial to some aspects of autism behavior in some patients (Kolmen, Feldman et al. 1995; Riddle, Bernstein et al. 1999; Elchaar, Maisch et al. 2006) but not all behaviors (Zingarelli, Ellman et al. 1992; Willemsen-Swinkels, Buitelaar et al. 1995; Feldman, Kolmen 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, King et al. 2001). Valproate has several mechanisms of action and is known to reduce stimulated, but not basal, cAMP levels (Montezinho, Mork et al. 2007). Mice exposed to valproate during development exhibit functional deficits that parallel autism (Ingram, Peckham et al. 2000; Wagner, Reuhl et al. 2006). The impact of valproate on the functional overconnectivity of cortical microcircuitry (Rinaldi, Silberberg 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, Yamagata et al. 2006; Siu, Lam et al. 2006). A case study (Horvath, Stefanatos 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; Sturmeay 2005; Williams, Wray 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, Blasi 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, Zhong 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.

cAMP Therapy

Treatment through the cAMP cascade may be beneficial in FX. Cyclic AMP was thought to regulate FMRP expression through an FMR1 enhancer (Hwu, Wang 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, Nicholls 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, Boyett-Anderson et al. 2004) and hypersensitivity to stress (Barad, Bourchouladze 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, Hasanat et al. 1999) to improve FX behavior. For example, the mood stabilizer lithium increases cAMP production (Chen, Hasanat et al. 1999) and is known to improve FX *Drosophila* memory behavior, as have group II mGluR antagonists which elevate cAMP levels (McBride, Choi 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, Huber 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, Padgett 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, Bevilacqua 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-Cyclopentylloxy-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, Vogt et al. 2002; Pace, Hu et al. 2007) of reduced numbers of FX dendritic glucocorticoid receptors (Miyashiro, Beckel-Mitchener et al. 2003) involved in hypothalamic-pituitary-adrenal (HPA) regulation (Hessl, Rivera et al. 2004). A clinical trial of rolipram reported acceptable tolerance levels at clinically relevant doses (Fleischhacker, Hinterhuber et al. 1992). In clinical and preclinical trials, the main human PDE4 inhibitor side effect, thought to be due to PDE4D activity (Robichaud, Savoie et al. 2002; Robichaud, Stamatiou et al. 2002; Conti, Richter 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, Zhang 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, Bourchouladze 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 (Bourchouladze, Lidge 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, Huang 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.

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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Figure 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.