ZINC-CONTAINING AMYGDALOID EFERENCE TO MEDIAl
PREFrontAL CORTEX: HISTOCHEMICAL CHARACTERIZATION AND
IMPLICATIONS IN DEVELOPMENT

Presented to the Department of Biology
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ABSTRACT

It has been demonstrated that the basolateral nucleus of the amygdala contains pyramidal neurons that project to medial prefrontal cortex, the rat correlate of anterior cingulate cortex in humans. It also has been observed that this nucleus contains neurons enriched with synaptic zinc that appear to have pyramidal cell bodies. In the present series of experiments, it was demonstrated using retrograde tracing with FluoroGold and autometallographic labeling of zinc-enriched neurons that a large percentage of those pyramidal neurons projecting to medial prefrontal cortex contain synaptic zinc. Preliminary findings indicate that the majority of these zinc-enriched neurons do not innervate medial prefrontal cortex until after post-natal day 38 of the rat. The discovery of these neurons, and their late development, has potentially important implications in psychiatric disease, particularly schizophrenia. It was also determined that future experiments to analyze the development of this cortico-limbic pathway would require a means to chronically administer psychoactive agents to the postnatal amygdala while causing minimal trauma. To achieve this end, a chronic infusion system was devised utilizing a 50 μm diameter microcannula to deliver agent driven by a mini-osmotic pump.
INTRODUCTION

Schizophrenia is a psychiatric illness affecting approximately 1% of the population and is characterized by disordered thought, blunting of affect, rigid motor patterns, delusions, and often hallucinations. This prevalent and debilitating disease represents one of the most complex challenges facing both society and clinical neuroscience research. Though the symptoms of schizophrenia can be muted pharmaceutically, drugs used to treat this disorder often have unpleasant side effects and, furthermore, treat only a subset of symptoms. Individuals suffering from schizophrenia are often under medical care for life. Additionally, they constitute a large percentage of the homeless population.

The long-held belief that the etiology of schizophrenia is due, in part, to a genetic abnormality is based upon studies demonstrating that the disorder shows inheritance patterns in families. Relatives of schizophrenic individuals are much more likely to have this psychiatric illness or related symptoms than the general population. However, the pathogenesis of this disease does not appear to be due to genes alone. This idea is derived from behavioral genetic twin studies demonstrating that the concordance rate for schizophrenia in monozygotic twins is only about 46% (Tsuang, 2000). Children with two schizophrenic parents also have a 46% risk of inheriting this disease, and the risk for siblings of an affected individual is 10% (Tsuang, 2000). This indicates that the pathoetiology of schizophrenia must have both genetic and environmental components. To date, there have been many theories, but no conclusive evidence showing how, biologically, this combination of internal and external factors induce those psychiatric diseases with partial genetic inheritance.

It has been theorized that environmental stress experienced during development—including trauma in the womb due to a mother’s illness, a difficult birth, and stressful events in adolescence—could cause alterations in gene expression and neural circuitry that could subsequently trigger the onset of schizophrenia in those genetically predisposed to the disease (Keshavan, 1999). This idea directs scientific focus to brain structures and pathways that do not mature fully until adolescence and adulthood, since neural function is most plastic, and perhaps more affected by environmental influences, while regions have not yet settled in their final topological
state (Brown and Dyck, 2003). For this reason, the efferent fibers from basolateral amygdala (BLA) to anterior cingulate (ACCx), sometimes called the amygdalofugal fibers, should be studied with a cognizance of implications in schizophrenia, since it has been demonstrated that the majority of axonal growth in this projection pathway occurs during and after the rat post-weaning period (Cunningham et al., 2002). From a functional standpoint, the fact that this late-developing pathway projects to ACCx is particularly interesting. This structure is situated between limbic structures and neocortex and forms a large number of connections with both. Functionally, the ACCx has a role in emotional learning, expression and evaluation of affect, and motor initiation. Disruption of this brain region results in inappropriate affect, muted emotional response, and impaired initiation of movement (Devinsky et al., 1995). These losses of function are frequent symptoms of schizophrenia and are often labeled its “negative” symptoms.

The ACCx is a site of numerous cytoarchitectural alterations in schizophrenic brain, as is demonstrated by a number of postmortem studies. Many of these abnormalities have been observed in the GABA system. For example, it has been shown that there is a significant decrease in nonpyramidal neurons within layer II of the ACCx (Benes et al., 1991) and, more recently, a decrease in parvalbumin-immunoreactive neurons, which represent a subset of GABAergic interneurons (Beasley and Reynolds, 1997). In addition to the GABAergic cell loss in the ACCx, there is a decrease in the rate-limiting enzyme for GABA production, glutamate decarboxylase, that is often used as a histological marker for GABAergic interneurons (Benes et al., 2000). Moreover, there seems to be a compensatory increase in binding to GABA\textsubscript{A} receptors found in this region (Benes et al., 1996). These findings point toward an overall decrease in GABA function in the schizophrenic ACCx.

It has been proposed that this decrease in GABAergic interneurons in ACCx might be the result of excitotoxic cell death triggered by an external stressor. Experience-driven stress causes the body to release stress hormones, such as glucocorticoids. This hormone, in combination with excessive glutamate release, have been shown to result in excitotoxic cell death (Coyle and Puttfarcken, 1993). GABAergic interneurons are particularly susceptible to excitotoxic injury mediated via the kainate subtype of
glutamate receptors, and glucocorticoids appear to potentiate the excitotoxic effects of kainic acid (Stein-Behrens et al., 1992). These observations are interesting, given that layer II of ACCx receives a massive input of glutamate-immunoreactive fibers, which are suggested to originate from the BLA. Furthermore, these fibers are significantly increased in schizophrenia (Benes et al., 1992). The increase in excitatory activity, which would be expected to result from an increase in glutamate immunoreactive fibers, could play a role in the hypothesized excitotoxic injury inflicted on GABA cells in schizophrenic brain. Moreover, the amygdala plays a prominent role in the experience and expression of fear and anxiety (LeDoux, 2000), and stress has been reported to increase activity within this brain region (Stamp and Herbert, 2001). This could exacerbate any excitotoxicity resulting from the postulated increase in amygdalofugal fibers.

**Communication in the Amygdalofugal Pathway**

The substances used by neurons to communicate in neural circuitry serve as important components of their identification and classification. They allow one to learn not only which cells can intercommunicate, but also the nature of the conductance that pre-synaptic neurons can induce in post-synaptic neurons during these communications. A single neuron can express a large variety of receptors, thereby allowing it to understand a variety of signals. However, the expressive abilities of a single neuron are considered to be more limited than its receptive abilities. Neurons are thought to express themselves with a single dominant neurotransmitter, and sometimes a co-transmitter, such as nitric oxide or ATP. Identifying the neurotransmitter of a neuron indicates if it is excitatory, inhibitory, or if it modulates the receptive abilities of the post-synaptic neuron. Though glutamate is the major excitatory neurotransmitter in the central nervous system, it is also an abundant amino acid, often making it ambiguous to determine histochemically whether a set of neurons are glutamatergic. For this reason, the neurotransmission properties of the projection neurons from the BLA to the rat homologue of the human ACCx, medial prefrontal cortex (mPFC), though purported to be glutamatergic, are still largely unknown.
While some studies have supported the notion that amygdalo-cortical connectivity is glutamatergic, this evidence remains equivocal. For example, Golgi-staining has demonstrated that amygdalar projection neurons are predominantly pyramidal, which are generally considered excitatory (McDonald, 1992). In addition, both glutamate and aspartate, which are excitatory amino acids, show high levels of immunoreactivity in these neurons (McDonald, 1996). They also show the colocalization of glutamate and tubulin fibers, which are used much like railways for axonal transport, indicating that this amino acid might be transported down the axon to serve as a neurotransmitter (McDonald et al., 1989). That this pathway is glutamatergic remains only putative, however, mainly due to the well-established fact that stimulation of the BLA leads to overall inhibition of mPFC (Matsuda and Fujimura, 1995; Perez-Jaranay and Vives, 1991). Though it has been suggested that this inhibition reflects the intermediary actions of GABAergic interneurons, this interaction has not been demonstrated cytologically.

Another means of further characterizing the signaling properties of the amygdalofugal fibers is by demonstrating that these neurons contain synaptic zinc. There is a subset of glutamatergic neurons sometimes referenced as “gluzinergic”, because their synapses contain both glutamate and zinc filled vesicles that are released via exocytosis in a calcium and impulse-dependant manner (Howell et al., 1984). Almost all neurons that contain zinc-filled vesicles are glutamatergic (Frederickson et al., 2000). This indicates that the localization of zinc-containing vesicles to synapses formed by amygdalofugal fibers within mPFC would indicate that these efferent fibers have a high probability of being glutamatergic. There are few exceptions to this trend, as the only non-glutamatergic neurons known to contain synaptic zinc are subsets of GABAergic inhibitory neurons discovered in the cerebellum and spinal cord (Wang et al., 2002). However, it is important to note that these inhibitory neurons containing synaptic zinc are present in regions of the nervous system that are known to have signaling properties that are often quite different from neurons within cerebral cortex and other “more-evolved” structures. The cerebellum, for example, contains networks of excitatory GABAergic interneurons (Chavas and Marty, 2003). In addition, neurons of
the spinal cord use acetylcholine as their main excitatory neurotransmitter rather than glutamate.

It is documented that a large proportion of the neurons that populate the BLA contain synaptic zinc (Christensen and Geneser, 1995). This zinc-enriched population of neurons comprise about 10% of the neurons in the BLA, most having the large "spiny" cell body type characteristic of pyramidal projection neurons (Christensen and Geneser, 1995). Although these neurons have not been positively associated with the projections that the basolateral amygdala sends to medial prefrontal cortex, given the large number of both types of neurons in the nucleus, it was hypothesized that at least some of the projection neurons are zincergic.

The identification of neurons projecting from the BLA to mPFC has several implications: it is a step towards positively identifying these neurons as glutamatergic and thus excitatory. In addition, it adds new dimensions to what is known about the signalling properties of this pathway. It has been demonstrated in cultures of cortical neurons that both excesses and deprivation of zinc can decrease cell survival (Chen and Liao, 2003). Zinc also has the ability to modulate the conductance of several glutamate and GABA receptors, adding an altered intonation to normal glutamatergic neurotransmission (Takeda et al., 2004). Instances of ischemic and excitotoxic insult and injury to the central nervous system cause massive amounts of zinc to be released, inducing cell death by way of both apoptosis and necrosis (Suh et al., 2000).

Zinc present in the neuronal boutons is considered “free” because it is not bound to any protein and is therefore the only zinc that is available for precipitation and visualization using histochemical techniques (Frederickson et al., 2000). All other zinc in the central nervous system is bound to enzymes, transcription factors, peptides, and metallothionein transport proteins. This makes it simple and reliable to identify zinc-enriched neurons, as free zinc can be precipitated using either sulfide or selenide salts, injected either intraperitoneally or directly into the brain (Frederickson et al., 2000). The location of the zinc sulfide or zinc selenide clusters that are deposited as a result of zinc precipitation in neurons can be controlled by varying the amount of time that the animal survives after injection with a sulfide or selenide salt. After one hour, the zinc salt clusters will remain in the axon termini (Christensen and Frederickson, 1998). After 24
hours, these zinc salt clusters can be transported retrogradely via axon transport mechanisms to the neuron soma (Christensen and Frederickson, 1998). The use of retrograde transport to carry these crystals has been verified by the ability of the cell transport inhibitor, colchicine, to block staining of the cell soma due to the precipitation of a zinc salt (Slomianka et al., 1990). Both forms of zinc clusters can be visualized using a silver salt developer for either Timm’s staining or autometallographic selenide (Neo-Timm’s) staining techniques (Danscher, 1981).

In addition to evaluating the signaling properties of the efferent projection neurons of the BLA, it is often necessary to make this amygdafofal pathway visible, so that one can identify it anatomically. Two means of evaluating the structural growth and development of efferent axons from the basolateral amygdala are anterograde and retrograde tracers. Anterograde tracers, such as biotin-dextran amine (BDA) and biocytin, allow one to visualize the location and density of fibers projecting from a given region by injecting the tracer into the region where the cell somas reside. The tracer is then anterogradely transported up the axon by the same molecular mechanisms of transport that carry vesicles of neurotransmitter (Figure 1A). This methodology led to the discovery that axons originating in the basolateral amygdala continue to innervate the mPFC until P60, when the rat reaches adulthood and the greatest increases in fiber growth from the BLA into mPFC during rat adolescence occur within cortical layers II and V (Cunningham et al., 2002) (Figure 1A). These layers are also those with the greatest synaptic density overall in mPFC (Bacon et al., 1996) and they demonstrate the strongest current in cell recordings when the amygdala is stimulated (Matsuda and Fujimura, 1995). Retrograde tracers such as Fluorogold (FG) and horseradish peroxidase (HRP) allow one to inject the tracer into regions such as mPFC where the axon takes up these molecules and carries them retrogradely within the axon back to the soma, using molecular transport mechanisms. This allows one to visualize distant neural somas sending projections to the region of injection (Figure 1B). Unlike anterograde tracing, this makes it possible to count the number of cells that project to the region and also conduct double-staining experiments to analyze the properties of the projection neurons, including its modes of neurotransmission. In the present experiments, rat mPFC was filled with the retrograde tracer FluoroGold. Subsequently,
Figure 1: Use of anterograde and retrograde tracing to characterize efferent projection neurons from the BLA to mPFC. A, A schematic diagram of anterograde tracing from the BLA to mPFC. Beneath this diagram are photomicrographs taken, with permission, from a previous study (Cunningham et al., 2002) showing efferent fibers in mPFC labeled with the anterograde tracer biocytin. As illustrated, the majority of efferent fibers from the BLA to mPFC innervate layers II and V. B, A schematic diagram of retrograde tracing from mPFC to the BLA. In this study, we aim to fill mPFC with the anterograde tracer FluoroGold, with the aim to fill both layers II and V. Neurons sending projections to this region absorb FluoroGold and the neuron fills with fluorescent dye. As the BLA sends a multitude of projections to mPFC, this retrogradely labels many cell bodies in the BLA.
animals were injected intraperitoneally with sodium selenite and sacrificed after 24 hours. Coronal brain slices were processed histologically using autometallographic techniques, so that cells in the BLA that project to mPFC and neurons containing synaptic zinc were individually identifiable and co-localization could be observed.

**Animal Models of Schizophrenia**

Another means of characterizing the signaling properties of these amygdalofugal fibers is to observe changes in this pathway when the amygdala is rendered hyperactive during adolescence. Recently, a "partial" model of schizophrenia was developed which mimics changes observed in post-mortem schizophrenic brain. This model suggests that changes incurred in the GABAergic system of the hippocampus (and potentially the ACCx as well) can be explained by chronic hyperactivity of the basal nuclei of the amygdala (Berretta et al., 2001). This evidence is supported behaviorally by the appearance of psychosis in cases of seizure activity originating in the amygdala (Takeda et al., 2001). Picrotoxin blocks chloride conductance in GABA-ligated channels and is often used to induce localized seizures in studies of epilepsy because it can cause hyperexcitability in regions of the brain where it is administered (Berretta et al., 1997). Preliminary evaluation using a single injection of picrotoxin to cause disinhibition of the amygdala shows that it induces many of the changes observed in postmortem tissue (Berretta et al., 2001).

Thus far, only the hippocampus has been evaluated to verify that amygdalar disinhibition can induce changes that mimic schizophrenia, it is likely, however, that this model can account for some of the changes observed in the schizophrenic ACCx as well. For example, hyperactivity could increase the density of vertical fibers innervating mPFC by means of sensitization. Sensitization is a presynaptic increase in neurotransmission complementing increases in the activity of a neuron that was first characterized in *Aplysia* (Bailey and Chen, 1989). During sensitization, neurons respond to repeated excitatory input by morphologically increasing output via an increase in presynaptic varicosities (Bailey and Chen, 1989). It has been demonstrated that even single events of stress can cause the basolateral amygdala to show long-term sensitization in the responsiveness of the metabolic marker c-fos (Bruijnzeel et al., 1997).
The rat amygdala has also been observed to positively respond to activity in the behavioral paradigm of restraint stress by exhibiting apical dendritic sprouting, particularly in the regions corresponding to excitatory input, thus increasing its access to excitatory signals that allow the neuron to fire and release transmitter (Vyas et al., 2002). Other studies administering seizure-inducing drugs, such as kainite, have demonstrated that projection neurons from the CA1 region of the hippocampus can undergo activity-dependent axonal arborization (Perez et al., 1996). Hyperactivity within the amygdala during vertical fiber development could have a particularly strong effect on the growth of those axons. Furthermore, such hyperactivity could contribute to the net loss of interneurons observed in layer II of this cortical region via excitotoxic mechanisms.

It is now possible, using methodology developed here, to evaluate the effect on mPFC function that chronic hyperactivity of the adolescent basolateral amygdala might incur as it communicates through these late-maturing axons. In conjunction with analyzing the zinc signaling properties of the amygdalo-fugal fibers to mPFC, we have devised a chronic microcannulation system that would allow delivery of the GABA channel blocker, picrotoxin, to a discrete area while minimizing trauma to the region. This innovation utilizing a pulled borosilicate glass cannula with a diameter of 50μm is an improvement over the 26 gauge metal cannulas most commonly used for chronic cannulation. These larger cannulas create lesions in the brain nearing 1mm in width, which may be greater than the dimensions of the target area. Previous literature describes a 33 gauge chronic cannulation device that has a 200μm diameter (Williams et al., 1987), however the newly designed cannula designed herein has a surface area that is still 16 times less than the 33 gauge cannula. Using this new tool we plan to evaluate gene expression changes, apoptotic and excitotoxic cell death, glutamate release, and changes in zinc metabolism in mPFC due to picrotoxin-mediated disinhibition of the amygdala.
METHODS

Subjects. Adult male Wistar-Kyoto rats weighing between 250 and 450g were used for the colocalization of FluoroGold and zinc selenide labeled neurons in BLA. For the evaluation of the BLA in adolescence, male Wistar-Kyoto rats arrived at the facility at P28. Both adolescent and adult Sprague-Dawley male rats were used to test microcannulation methods. All animals were housed in a virus and antigen free facility on a 12 hour light/dark schedule with free access to food and water.

FluoroGold injections. All animals were anaesthetized with 87mg/kg ketamine and 13mg/kg xylazine administered intraperitoneally prior to stereotaxic surgery. 0.1μm of 2% FluoroGold (FluoroChrome, Inc., Englewood, CO) was injected intracortically to medial prefrontal cortex in the right hemisphere using a stereotaxic methodology and sterile technique. In adult animals, injections were made at the following coordinates, using bregma as reference: AP +2.7, L 0.25, V -4.4. In P30 animals, injections were made at the coordinates: AP +2.5, L 0.27, V -4.0. Subsequent to injections, wounds were washed with sterile saline, sutured, and antibiotic was applied topically.

Intraperitoneal delivery of sodium selenite. Seven days following FluoroGold injection and 24 hours prior to sacrifice, animals were injected interperitoneally with 8mg/kg of sodium selenite dissolved in distilled water.

Perfusion. Adult animals used for autometallography were perfused transcardially with a cold solution of 4% paraformaldehyde and 0.1% gluteraldehyde dissolved in phosphate buffer with a pH of 7.4. P38 animals and animals used to test microcannulation techniques were perfused transcardially, first with cold 0.1M phosphate buffered saline, followed by 4% paraformaldehyde dissolved in phosphate buffer. All perfusion solutions used at 4°C with a pH of 7.4.

Tissue Preparation. Rat brains were cryoprotected in a solution of 30% sucrose following perfusion. Adult brains used for autometallography were sectioned serially and coronally on a cryostat at a thickness of 30μm and thawed onto clean 5% gelatin-coated
glass slides. Adolescent brains used for autometallography were sectioned and mounted in the same manner as adult brains but were instead cut at 60µm to protect tissue integrity. Animals used to test microcannulation techniques were sectioned coronally at 40µm on a sliding microtome and tissue was stored in cold 0.1M phosphate buffered saline with 0.1% sodium azide antimicrobial agent before staining and mounting on gelatin or poly-lysine coated slides.

**Silver amplification autometallography procedure.** Cryostat cut sections placed on gelatin-coated slides were prefixed in 95% ethanol for 15 minutes, rehydrated, and air-dried. A silver lactate physical developer was prepared with minimal light exposure using glassware that was thoroughly cleaned with Farmer's reducer (9 parts 10% sodium thiosulfate + 1 part 10% potassium ferricyanide) prior to use. The physical developer contains 60mL protective colloid (50% gum arabic in deionized water), 10mL citrate buffer (25.5g citric acid monohydrate + 23.5 sodium citrate dihydrate in 100mL deionized water), hydroquinone as a reducing agent (0.850g in 15 mL of deionized water), and silver lactate (0.121g in 15mL of deionized water) as a donor of silver ions to zinc selenide clusters. The citric acid buffer was stored at 4°C for a period of up to two weeks or until it became cloudy. The hydroquinone and silver lactate solutions were prepared just prior to the development reaction, and the silver lactate solution was added last to the developing solution to start the reaction. Development took place in a dark box placed on an automatic shaker at room temperature for 50-90 minutes. After development, slides were exposed to a stop solution of 5% sodium thiosulfate for 12 minutes and then placed under running lukewarm tap water for 45 minutes to remove development solution. Slides were then dipped in a bath of distilled water followed by a bath of 10% Farmer's reducer solution to remove non-specific background. Slides were again dipped in a bath of distilled water, post-fixed in 70% ethanol for 30 minutes, dehydrated and cover-slipped with Cytoseal® coverslipping solution.

**Cannula and osmotic pump assembly.** These experiments utilized two microcannula designs. In the first design, a pulled borosilicate glass micropipet with a 50µm diameter tip was fitted to a truncated 26 gauge steel cannula, using light-curing adhesive. In the
second design, a pulled micropipet with a 50μm diameter tip was bent over a heated wire to a 90 degree angle to enable direct attachment to the osmotic pump tubing. 100μL Alzet® osmotic pumps with an 0.25μL/hr flow rate were filled with saline-diluted india ink or Dil, using a 1cc syringe for injection. The osmotic pump was then attached in an air-tight manner to a stainless steel tube and nylon catheter. The pump was then allowed to flow in vitro for 24 hours. The catheter was then truncated 2cm from the osmotic pump and attached to either a standard 26 gauge steel cannula or a microcannula and sealed using a light-curing adhesive. The pump was allowed to flow in vitro overnight while attached to the cannula before use.

**Placement of chronic cannulas and osmotic pumps.** All animals were treated with ketamine anesthesia prior to stereotaxic surgery. Cannulas were placed stereotactically into basolateral amygdala (AP -2.3, L 4.5, V -7.4) or striatum (AP +0.2, L 3.2, V -5.2) and secured using DenMat® dental cement. Subsequent to injections, wounds were cleaned with sterile saline, sutured, and antibiotic was applied topically. Animals were sacrificed using transcardial perfusion 2-5 days following cannula placement.
RESULTS

Intracortical injections of FluoroGold (FG) to the base of the infralimbic cortex (IL), filled both the IL and Cg3 regions of medial prefrontal cortex. This ensures that all neurons sending axons to mPFC take up FG and backfill to the soma. Neurons in the BLA show robust retrograde labeling, as observed in fluorescent photomicrographs (Figure 2).

Intraperitoneal injection of sodium selenite resulted in retrograde labeling of neurons that contain synaptic zinc. High power photomicrographs allowed visualization of the silver-amplified clusters of zinc selenide (ZnSe) that accumulate in zincurgic cell somas (Figure 3). These clusters, resembling dark brown specs, were observed to accumulate in the neuron soma, primarily along the periphery of the neuron.

Overlaying light photomicrographs depicting ZnSe clusters in the BLA (Figure 4A) with fluorescent photomicrographs showing FG-filled neurons in this region (Figure 4B), revealed a multitude of cells that contain both FG and silver-amplified ZnSe clusters (Figure 4C). Neurons exclusively containing FG and neurons exclusively containing ZnSe clusters were also readily observed. This demonstrates that there are two populations of neurons in the BLA that project to mPFC: those that contain vesicles of synaptic zinc and those that do not.

OpenLab 3.0.7 computer-assisted quantification software was utilized to quantify cells in three equivalent 30 µm coronal slices of the adult BLA for three subjects. Counts of neurons doubly labeled with FG and ZnSe clusters, single labeled with FG, and single labeled with ZnSe are depicted in Table 1. The slices that were evaluated at represented sections approximately 2.30, 2.80 and 3.30mm posterior to bregma. It was demonstrated that between 28 and 39% of all amygdalofugal neurons to mPFC contain synaptic zinc, with the least zinc labeling in the most anterior section and the most zinc labeling in the most posterior section. The associated standard deviations from these values ranged from 2 to 6 %. Illustrations of the coronal sections from which these cell counts were obtained are depicted in Figure 5.

We undertook a pilot developmental study to determine if efferent fibers with and without synaptic zinc innervate mPFC asynchronously by evaluating neurons co-labeled
with FG and ZnSe clusters in the adolescent (P38) rat. It is during this time in adolescence that the amygdalofugal fibers show robust growth into mPFC (Cunningham et al., 2002). Preliminary results show a drastic reduction in the proportion of FG-filled neurons that co-label with ZnSe in the brain of adolescent rat (Table 2, Figure 6). This percentage is more than 5 standard deviations less than in equivalent sections evaluated in the adult rat. The one-tailed Dixon outlier test was used to demonstrate significant deviance from the adult normal curve for FG double-labeled cells at 2.30 and 2.80mm posterior to bregma. Also observed were significant deviances in FG labeled cells at 2.80mm posterior to bregma and in ZnSe labeled cells 3.30mm posterior to bregma.

Figure 7A depicts the standard 26 gauge chronic cannula which produces significant damage in the central nervous system of the rat, creating lesions extending to more than 1mm in diameter (Figure 9A). Newly designed 50μm diameter pulled borosilicate microcanulas were subjected to both in vitro and in vivo tests. In vitro tests demonstrated proper fluid-flow through the microcannula (Figure 8). The first in vivo test of a microcannula also caused lesioning, though it was characteristic of damage due to increased fluid pressure at the tip rather than trauma induced by the size of the cannulation device. When an improved version of this cannula (Figure 7C) was attached to an 0.25mL/hr osmotic pump with a flow rate reduced by approximately half, a 50μm cannula allowed the focal delivery of Dil without damage (Figure 9C).
**Figure 2:** Retrogradely-labeled amygdalofugal neurons projecting to mPFC. **A.** A schematic diagram of retrograde tracing from mPFC to the BLA. As illustrated, the tracer must fill mPFC broadly to cover layers II and V. It also must follow the injection pipet track dorsally to fill both the Cg3 and IL regions of mPFC. **B.** A representative injection of FluoroGold to mPFC from the collected data. As observed in the fluorescent photomicrograph on the left, the injection fills mPFC. Fluorescent microphotograph images on the right show cell somas retrogradely labelled with FluoroGold throughout the BLA.
Figure 3: Zinc-enriched neurons (ZENs) as observed using silver amplification autometallography to amplify precipitated zinc selenide clusters in the BLA for visualization.
Figure 4: Zinc selenide and FluoroGold labeled neurons in the adult basolateral amygdala. "I" indicates a neuron labeled with zinc selenide alone. "II" indicates a neuron labeled with FluoroGold. "III" indicates a neuron co-labeled with both FluoroGold and zinc selenide. A, light photomicrograph showing only zinc selenide labeling. B, fluorescent photomicrograph of the section depicted in A, showing only FluoroGold labeling. C, overlaid light and fluorescent photographs (from A and B).
### TABLE 1: FluoroGold and Zinc Selenide Labeled Neurons in the Adult Basolateral Amygdala

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Table 1 contains the raw FluoroGold (FG) and zinc selenide (ZnSe) cell counts, means, and standard deviations for three adult animals in three coronal 30μm coronal slices of basolateral amygdala. Also in this table are calculations of the percent FG labeled cells also labeled with ZnSE and the percent of ZnSe labeled cells also labeled with FG along with their respective means and standard deviations.
Figure 5: Overlaid light and fluorescent photomicrographs of the adult BLA. Coronal sections were sliced on a cryostat at 30μm. Insets in the lower right corners of each slice depict the coronal section from which the photomicrograph was taken. The area representing the BLA is outlined in red. A, representative section corresponding to cell counts taken at 2.30mm posterior to bregma. B, representative section corresponding to cell counts taken at 2.80 posterior to bregma. C, representative section corresponding to cell counts taken at 3.30 posterior to bregma.
TABLE 2: FluoroGold and Zinc Selenide Labeled Neurons in the P38 Basolateral Amygdala

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<th>Percent Double-labeled</th>
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</thead>
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<tr>
<td></td>
<td>ZnSe + FG</td>
<td>Total FG</td>
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<tr>
<td>60μm Coronal Slice</td>
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<tr>
<td>Adjusted to 30μm</td>
<td>0.5</td>
<td>7</td>
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<tr>
<td>r10 (Dixon Outlier Value)</td>
<td>0.600</td>
<td>0.638</td>
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<tr>
<td>% Increase in Adulthood</td>
<td>5933%</td>
<td>279%</td>
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<td>60μm Coronal Slice</td>
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<tr>
<td>r10 (Dixon Outlier Value)</td>
<td>0.863**</td>
<td>0.764*</td>
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<td>% Increase in Adulthood</td>
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<td>% Increase in Adulthood</td>
<td>2967%</td>
<td>327%</td>
</tr>
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* p<0.1  ** p<0.05

Table 2 contains raw data and statistical analysis of a preliminary quantitative evaluation of FluoroGold (FG) and zinc selenide (ZnSe) labeling in the BLA of a single adolescent (P38) rat. The Dixon outlier test was used to evaluate the variance of the adolescent from the adult cell counts.
Figure 6: Zinc selenide and FluoroGold labeled neurons in the P38 basolateral amygdala. I corresponds to a neuron labeled with zinc selenide alone. II corresponds to a neuron labeled with FluoroGold. III corresponds to a neuron co-labeled with both FluoroGold and zinc selenide. A. A light photomicrograph showing only zinc selenide labeling. B. A fluorescent photomicrograph of the section depicted in A, showing only FluoroGold labeling. C. The overlaid light and fluorescent photographs (from A and B). Note that the neuron to the upper left of II is not double labeled, as the FluoroGold and zinc selenide labeled neurons that appear to overlay in C have different shapes.
Figure 7: Several chronic-use cannula designs A. The standard 26 gauge steel cannula. B. A microcannula design in which a pulled borosilicate glass pipet is attached to a truncated standard cannula pedestal. C. In this updated version of the chronic microcannula displayed in B, a pulled borosilicate glass cannula is bent at a 90 degree angle for direct attachment to the osmotic pump.
Figure 8: The *in vitro* experimental arrangement to test the flow of drug through the cannula tip. The dye-filled osmotic pump was suspended in physiological saline solution. Plastic tubing connected the osmotic pump to a microcannula which delivered dye to a saline-filled receptacle. **A.** The *in vitro* test at the beginning of evaluation. **B.** After several hours *in vitro* dye may be visualized in the receptacle solution, indicating flow due to the action of the osmotic pump.
Figure 9: Comparison of tissue damage induced by several cannula designs. A. The standard 26-gauge steel cannula creates large areas of damage due to increased pressure at the cannula tip. B. Chronic microcannulation at 0.25 μl/hr causes a lesion due to increased pressure at the cannula tip. C. Chronic microcannulation at 0.125 μl/hr can effectively distribute dye to a discrete area of tissue with minimal damage.
DISCUSSION

That neurons within the amygdalofugal pathway contain synaptic zinc is interesting for three major reasons. First, it shows that this pathway has a new potential means of cell-to-cell communication, particularly one that reinforces the identification of these efferent neurons as glutamatergic. Second, the identification of two neuronal types in this late forming projecting pathway could make this pathway particularly useful in studying patterns of postnatal axonal development. Third, it opens the possibility that the ability of synaptic zinc to induce cell death and modulate the activity of some glutamate and GABA receptors, such as the N-methyl d-aspartate (NMDA) glutamate receptor, could, when activated abnormally, lead to cell composition and receptor activity changes seen in the ACCx in psychiatric disorders such as schizophrenia. This information, paired with the development of a new means of chronically administering drug to small regions of the rat central nervous system, opens many new avenues of investigation for this psychiatrically and developmentally important neural pathway.

Zinc Neurotransmission

In zinc-enriched neurons (ZENs), zinc is packaged densely into small vesicles with the help of zinc transporting proteins called ZnTs. The ZnT-3 version of this protein is found exclusively in the brain and testes and is thought to be the sole means of packaging zinc into synaptic vesicles. Five percent of the total zinc concentration in the brain is present in these vesicles, a proportion similar to the amount of glutamate used as a neurotransmitter (Frederickson et al., 2000). When prompted by calcium influx due to a propagating action potential, these vesicles, like glutamate vesicles, fuse to the bouton membrane, and their contents are exocytosed into the synaptic cleft (Howell et al., 1984).

Once zinc is released into the synaptic cleft, its role is less defined than that of most neurotransmitters. This is because zinc does not have its own receptor, but rather influences the activity of many ion channels and other proteins allosterically. In this sense, zinc can act in the nervous system much like calcium, as a voltage-influencing
cation, second messenger, and allosteric regulator (Frederickson et al., 2000). One receptor on which the zinc cation has a strong effect is the NMDA receptor (Christine and Choi, 1990). Zinc can allosterically block some NMDA receptors bound to glutamate when present in quantities as low as the nanomolar range, which could have a large effect on synaptic plasticity due to coincidence detection, such as long-term potentiation (Paoletti et al., 1997). This channel blockage is not voltage-dependant, like that of another divalent cation, magnesium. Conversely, zinc seems to potentiate AMPA receptor currents, but with a much smaller effect (Rassendren et al., 1990). It has also been demonstrated that zinc has the ability to block GABA<sub>A</sub> channels (Takeda et al., 2004). It is still controversial whether zinc is released in quantities large enough to affect post-synaptic receptor conductance during normal activity, in the absence of ischemia, seizure, or other injury. Still, recent findings showing that there may be less zinc released from ZENs than previously calculated noted that even these low levels of zinc in the synapse are capable of blocking NMDA channels due to their high affinity for the receptor (Kay, 2003).

During instances of ischemic (Sorensen et al., 1998) and seizure-driven (Takeda et al., 2003b) injury, the release of zinc can have drastic effects. In such situations, massive dumping of zinc from axon terminals results in high levels of extracellular zinc. Additionally, zinc is released from the carrier protein metallothionein-III (Lee et al., 2003) which tends to cluster in regions with large numbers of zinc-enriched boutons (Masters et al., 1994). This carrier protein is produced in neurons that release vesicular zinc, though the mature protein localizes in nearby astrocytes, suggesting that it plays a role in carrying circulating zinc to the neurons (Hidalgo et al., 2001). Metallothioneins can carry up to seven zinc ions in a non-cooperative manner (Ebadi et al., 1995). An excess of zinc can result in both apoptosis or excitotoxicity due to the passage of zinc through zinc permeable channels, including NMDA, AMPA, kainate, and some voltage gated calcium channels. Once zinc enters the neuron it activates both apoptotic and necrotic cascades by up-regulating the p75 neurotrophin receptor and inducing PKC-mediated second messenger cascades respectively (Koh, 2001). It has been demonstrated that the chelation of synaptic zinc decreases these processes that lead to cell death after
ischemic injury by up to 80%, strongly suggesting that zinc is one of the primary effectors of injury-induced neuronal death (Koh, 2001).

The role that synaptic zinc plays during non-adverse circumstances has been explored in knockout studies in which the ability on neurons to package zinc into vesicles is disrupted. In ZnT3-null mice, it was found that these mice are particularly prone to seizures (Cole et al., 2000). Synaptic zinc seems to have an overall stabilizing effect on normal neurotransmission by decreasing stimulus evoked glutamate release (Takeda et al., 2003a). Zinc-deficient mice also have decreased extracellular levels of GABA (Takeda et al., 2003a). Though these knockouts do not demonstrate unprovoked epileptic activity, they show a consistent increase in seizure susceptibility as induced by kainic acid. Mice with a knockout of the gene coding for the metallothionein III zinc carrier protein also show this increased susceptibility to kainate-induced seizures (Erickson et al., 1997). These knockouts do not seem to have any behavioral deficits, leading some to argue that synaptic zinc has little or no effect on behavior (Cole et al., 2001). Currently, there are no means with which to study the effect of otherwise normal neurons carrying excessive levels of synaptic zinc, nor a means to evaluate subtle behavioral changes that might be found in humans lacking vesicular zinc. The dearth of information regarding the function of synaptic zinc under non-adverse conditions makes ruling out a role for synaptic zinc in complex behavior premature.

Though much is uncertain regarding the transmitting abilities of synaptic zinc, it is very likely that it changes the “tone” with which glutamatergic neurons communicate. The finding that many zincergic terminals in mPFC originate in the BLA puts their parent neurons in a minority of cortico-limbic zincergic projection neurons, as most zincergic terminals in cortex are intracortical (Land and Akhtar, 1999). Further research should elucidate the exact nature of the “tone” that the amygdala sends this emotionally-implicated cortical region.

**Implications in Development**

Given that the majority of efferent projections from BLA to mPFC grow during the post-weaning period of development and the present discovery that there are at least two populations of neurons in this pathway that have different neurotransmission
abilities, we designed a preliminary investigation to determine whether these neurons innervate mPFC asynchronously. The data show promise for demonstrating that the population of neurons that innervate rat mPFC before post natal day 30 have a much lower proportion of zinc enriched terminals than the projections comprising the adult efferent pathway. The P38 animal evaluated for co-localization of ZnSe clusters and FG-labeled cells in the BLA (with injections of the retrograde tracer FG made at P30) showed only approximately 10% of FG-labeled neurons co-labeling with ZnSe clusters. Meanwhile, the adult animals had an average of 37% of FG-labeled neurons in the BLA co-labeling with ZnSe. The average standard deviation for these counts was 5%, creating a potential difference of 5 standard deviations in percent of co-labeled neurons projecting to mPFC between the adult and adolescent animal. Also, in the anterior two of the three coronal sections, FG double-labeled cell counts were significantly lower than the normal distribution of the adult counts as evaluated using the Dixon outlier test. This strongly indicates that very few neurons that project to mPFC from the BLA before P30 are enriched with synaptic zinc.

One issue to address in a pilot study that has only one subject is whether this trend, though drastic, may have been induced by experimental error. Though it may appear that there could be experimental error due to the lesser number of ZENs in the adolescent amygdala, any systematic error introduced into the data of this pilot study is unlikely to affect the proportions of neurons in the BLA that co-label with FG and zinc. One might argue that while FG labeling is lower in adolescents, as expected due to the late growth of this pathway, there may be reduced zinc labeling to tissue preparation that would reduce co-labeled cells. Looking at the percent increase of double-labeled neurons in the BLA in adulthood versus the increase in all zinc-labeled cells, there is a 4250% increase in double-labeled neurons in adulthood versus an increase of 290% in adulthood of zinc-labeled cells in the three coronal sections of BLA. Thus, any erroneous lack of zinc labeling cannot solely account for the observed lower numbers of ZENs that project to mPFC during adolescence. If there was a random reduction in zinc staining, and the innervation of mPFC by both cell types was synchronous, this percent increase of the proportion of double-labeled cells would be the same as the percent increase of zinc-labeled cells in adulthood. It is necessary to also indicate that there
was a slight anterior-posterior trend in this percent increase of zinc labeled cells in adulthood, with the greatest difference in anterior sections and the least increase in posterior sections, so if there was any systematic error in the zinc stain for this adolescent subject, there is still a strong argument that most anterior zincergic projection neurons in the BLA project to mPFC after P30, if not those in the more posterior regions.

The finding that zincergic axons might follow non-zincergic axons in the development of a pathway of projection fibers would not be an unanticipated finding. There are many indications that zinc-enriched neural circuitry continues to grow and mature during postnatal development in rat. There are temporally consistent increases in levels of stainable zinc at the synapses of almost all brain regions containing zinc termini and these increases continue during postnatal development (Valente et al., 2002). It was also observed that increases in the zinc content of these terminals generally corresponded to the time of synaptogenesis and synapse maturation of the pathways projecting to the areas of interest (Valente et al., 2002). Regarding the development of new neurons containing synaptic zinc, it has been observed in the mouse, that the granule cell layer of the hippocampus continues to generate new ZENs until P28 (Slomianka and Geneser, 1997).

It is also not unreasonable to postulate that neurons not containing synaptic zinc and ZENs innervate mPFC in succession. There are several means by which projection neurons, such as those in the BLA, can target axons to distal regions such as mPFC. In the one mechanism, axons can respond to signals relayed by the target neurons in a graded, pre-programmed manner and use the extracellular matrix as a guide to its destination (Araujo and Tear, 2003). Neurons that follow this pathway are called pioneer neurons because they are the first neurons from a region to reach their destination. In a second mechanism of axon targeting, neurons that reach their destination later can follow signals conveyed by the pioneer neuron as a guide to the final target. There is evidence of the use of cell adhesion molecules (CAMs) in this guidance process, such as fasciclin (Araujo and Tear, 2003; Nassif et al., 1998). Naturally, the signals regulating postnatal axon targeting can depend upon activity level. For example, fibroblast growth factor, a molecule mediating postnatal neurite outgrowth
to cortex from the thalamus, shows activity-dependant abundance (Lotto and Price, 1995). As the amygdalofugal efferent pathway develops postnataley, it is most-likely susceptible to activity-dependant processes. This provides a potential mechanism by which zincergic innervation could be modulated during development.

**Neurotransmitter Models of Schizophrenia**

Much of what is known about the role of neurotransmitter and receptor function in various psychiatric disorders is based upon observations of the effects pharmacological agents have on behavior. For example, the dopamine hypothesis of schizophrenia, which postulates that dopamine hyperfunction comprises part of the underlying pathophysiology of this disease, is derived from the fact that antipsychotic drugs block the dopamine D2 receptor (Seeman, 1987). In addition, psychotropic “street drugs”, such as cocaine and amphetamine, activate dopamine release and create many of the “positive” symptoms associated with schizophrenia, such as hallucinations, delusions, and agitation (Seeman, 1987). However, because the negative symptoms of schizophrenia (including blunted affect and psychomotor retardation) are absent during dopamine hyperactivity, it is evident that this hypothesis cannot depict the full psychopathology of schizophrenia.

More recently it has been observed that drugs interfering with the glutamate system by blocking NMDA channels can produce the positive symptoms of schizophrenia, as well as many of the negative symptoms. These psychotropic drugs and anesthetic agents, including ketamine, MK-801, and phenylcyclohexide (PCP) can induce the symptoms of schizophrenia in a way that is often clinically indistinguishable from this psychopathology (Tsai and Coyle, 2002). Furthermore, these pharmacologic agents have been shown to result in cell death, as observed in the post-mortem tissue of schizophrenic individuals, within the cingulate cortex of rat brain (Bueno et al., 2003). Chronic, sub-anesthetic use of these drugs causes both the behavioral changes observed with this disorder and metabolic changes observed in schizophrenic patients, such as hypofrontality (Tsai and Coyle, 2002). This indicates that these NMDA channel blockers induce schizophrenia-like symptoms not only by their actions on the channel itself, but also by gene expression changes induced by NMDA hypofunction. It has also
been demonstrated that the use of ketamine by neuroleptic-free schizophrenics can increase the severity of symptoms, beyond the scope of behavior elicited by ketamine in normals (Malhotra et al., 1997).

The myriad of behavioral effects induced by NMDA blocking agents are reflective of the complexity of action of the NMDA receptor itself. This receptor has a role in synaptic plasticity that far exceeds its simple appearance as a ligand-gated ion channel. NMDA channels are heteromeric tetramers that generally contain a combination of glycine-binding NR1 subunits and glutamate-binding NR2A-D subunits. NR2A and NR2B are the most common subunits paired with NR1 in the central nervous system while NR2C and NR2D are primarily found in the cerebellum and the brain stem, respectively (Cull-Candy et al., 2001). There also appears to be a shift in the subunit composition of NMDA receptors during development because levels of the NR2A subunit exceed levels of NR2B from adolescence into adulthood (Law et al., 2003).

There are three conditions for the open state of the NMDA channel: the NMDA receptor must be bound to glutamate and glycine, and there must be an elevated membrane voltage to remove the voltage-dependant blockade of this channel by the divalent ion magnesium. Once the NMDA channel is open it has the ability to pass the monovalent ions sodium and potassium as well as the divalent and catalytically-important ion calcium. Because the opening of the NMDA channel is dependent upon coincident pre- and postsynaptic activity (the requirements for LTP induction), it is believed to contribute to Hebbian learning (Jaffe and Johnston, 1990). With the aid of the enzyme calcium/calmodulin dependant kinase II (CaMKII), the NMDA receptor is able to strengthen the synapse via the insertion of AMPA glutamate receptors into the post-synaptic membrane in a calcium-dependant manner (Barria et al., 1997).

It has been demonstrated that decreasing metabolic activity in the ACCx results in symptoms resembling the negative symptoms of schizophrenia (Devinsky et al., 1995). It has also been shown that blocking NMDA receptors can have the same effect (Tsai and Coyle, 2002). Thus, it is logical to postulate that malfunctioning of NMDA receptors in this region, leading to an overall decrease in metabolism, is a potential source of these symptoms in schizophrenia. In this regard, the nature of glutamatergic inputs to the ACCx is of interest. As the BLA sends a large quantity of likely
glutamatergic fibers to the ACCx, it could potentially contribute to the postulated NMDA receptor hypofunction in this area. It is therefore interesting to learn that a subpopulation of these projection neurons that innervate rat mPFC in late adolescence contain a co-transmitter which blocks NMDA channel currents, much like the psychosis-inducing drugs ketamine and PCP. The NMDA receptors located on GABAergic interneurons are most sensitive to NMDA blocking agents (Grunze et al., 1996), therefore it has also been suggested that the hypofunction of NMDA receptors in schizophrenia has a particularly strong affect on these neurons. This is an interesting prospect for the cortical BLA projection pathway because electrophysiological evidence indicates that these glutamatergic axons strongly innervate the inhibitory interneurons of mPFC.

There are several pieces of evidence that suggest synaptic zinc has the ability to exert actions similar to those of NMDA antagonists at this receptor. For example, like ketamine, zinc has the ability to block the NMDA receptor by reducing open frequency (Christine and Choi, 1990). Zinc exerts this effect via a high affinity, voltage-independent block of the channel by binding to the outside of the NR2A-D subunit (Choi and Lipton, 1999). In a low-affinity manner and voltage-dependant mechanism similar to the magnesium block on NMDA channels, zinc can situate within the channel pore and lower current amplitude (Christine and Choi, 1990). Unlike magnesium, zinc also has the limited ability to pass through the NMDA channel pore, thus current is not completely eliminated in these circumstances (Christine and Choi, 1990). It is useful to note that zinc does not block all NMDA channels with equal efficacy. It shows highest affinity for those receptors containing NR2A subunits and binds at a histidine-rich region on this subunit (Choi and Lipton, 1999; Paoletti et al., 1997). It is useful to note that NR2A knockout mice have also been described as an animal model of schizophrenia and that the ability of ketamine to induce hypnotic behavior in rats is weaker in these knockouts (Sato et al., 2004). Also, although the zinc high-affinity binding site is on the NR2A subunit, the oxidation state of several cystine-cystine disulfide bonds in distal regions (two on the NR1 subunit and one on the NR2A subunit) contribute to the ability of bound zinc to block NMDA-mediated current (Choi et al., 2001)

Zinc’s ability to modulate the function of the NMDA receptor has also been implicated in another psychiatric disease, clinical depression. Reduced ability of zinc to
bind to NMDA receptors has been observed in the hippocampus of the post-mortem brains of suicide victims (Nowak et al., 2003). In animal models of depression, including learned helplessness and forced swim, it has been observed that zinc supplementation can decrease depressive symptoms (Rosa et al., 2003). It has also been observed using the learned helplessness model that rats subjected to this series of tests have reductions in levels of a zinc transporter protein (Nakatani et al., 2004). Two other NMDA blocking agents, ketamine and MK-801, also show the ability to increase motivation and mobility in rats tested under the same experimental designs, but this ability is lost in the case of sub-threshold competition with zinc (Rosa et al., 2003). Recently, there have been promising clinical results that show significant improvement of depression in patients who add zinc to their normal antidepressant pharmacological therapy (Rosa et al., 2003). This leads to the reciprocal idea that in cases of NMDA hypofunction, as observed in schizophrenia, zinc chelation could have a therapeutic affect.

Other means of causing NMDA hypofunction in schizophrenia have been previously investigated. As both ketamine and PCP act on the glycine binding subunit NR1, there are some theories, though unproven in terms of gene expression, that in schizophrenia there is an abnormal expression of enzymes regulating the availability of glycine, such as serine racemase and d-amino acid oxidase (Coyle et al., 2003). Other endogenous antagonists of the NMDA receptor that have been suggested as the endogenous equivalent to ketamine in schizophrenia are kynurenic acid and N-acetyl aspartic acid (Coyle et al., 2003). Levels of kynurenic acid, a tryptophan metabolite, are increased in prefrontal cortex of schizophrenic brains, and there is a decrease in the degrading enzyme for N-acetyl aspartic acid (Schwarz et al., 2001; Tsai et al., 1995). Kynurenic acid, however, must be at high levels to block NMDA channels, and has a more primary role as a nicotinic acid channel blocker (Miller et al., 2004). N-acetyl aspartic acid levels are reduced in the ACCx in schizophrenic patients despite the reduction in levels of its degrading enzyme (Theberge et al., 2003). Like most studies showing the simple observation of changes in expression levels of a protein or abundance of a substance, there is a lack of explanation for these changes in expression levels and their regional character, so they could be secondary or
reactionary to the changes of most concern in the pathology of schizophrenia. In this case, the possibility of another modulator of NMDA channels at work is still open.

Zinc, however, as a co-transmitter, has a pre-determined localization of action that is restricted to the anatomical regions containing zincergic innervation. Abnormal release of zinc could also be explained by experience-dependant activity in the regions from which it is released. The ACCx is not the only brain region implicated in schizophrenia that receives strong zincergic input. Other areas of interest for schizophrenia research, such as the hippocampus, amygdala, cerebral cortex, nucleus accumbens, and olfactory bulb, all receive high levels of zincergic innervation (Valente et al., 2002). The zinc-rich innervation of the olfactory bulb, primarily originating from the hippocampus, is especially of interest in conjunction with changes seen in the schizophrenic ACCx. There is a sharp reduction in smell identification ability in schizophrenics that is easily testable, correlated with reduced volume of this region and conserved in many non-schizophrenic relatives in pedigrees containing many schizophrenic individuals (Moberg and Turetsky, 2003; Turetsky et al., 2003). This symptom is also positively correlated with the negative symptoms of schizophrenia associated with the hypofunction of ACCx, including social apathy and social dysfunction (Malaspina and Coleman, 2003). This correlation is significant, and reliable “smell tests” have been developed to help identify schizophrenics that present a strong negative character to their symptomology (Goudsmit et al., 2003). It has been suggested therefore that negative symptoms and disability of olfaction are mediated by the same substrate in schizophrenia (Goudsmit et al., 2003). It is logical, in this case, to speculate that the common substrate may be heightened zinc neurotransmission.

Microcannulation and Future Directions

The development of a chronic low-flow microcannulation system to induce chronic disinhibition of the basolateral amygdala during adolescence may be used to aid future evaluation of this model of zincergic hyperinnervation of ACCx in schizophrenia. In upcoming experiments, it will be necessary to verify that zincergic innervation from
the BLA to mPFC occurs during the later stages of development of this pathway. It will also be necessary to further verify that synaptic zinc and an anterograde tracer injected into the BLA, such as the fast tracer biocytin, coexist in the same boutons in mPFC by evaluation with electron microscopy. Then, chronic cannulation using the new microcannulation system will allow the induction of amygdalar hyperactivity by disinhibition with picrotoxin and will cause minimal damage to the region of the BLA. This creates the ability to evaluate changes incurred in mPFC by amygdala hyperactivity in a variety of ways including surveying changes in gene expression using RT-PCR, evaluating of changes in cell survival in mPFC, and monitoring levels of neurotransmitters such as glutamate, GABA, and dopamine in this region by using microdialysis.
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31


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