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**Changes in Connexin 32 Protein and Gene Expression Following Cocaine
Self-Administration**

by

Jennifer Monique Arnold, M.Sc.

A dissertation submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of
Doctor of Philosophy

Department of Psychology
Specialization in Neurosciences

Carleton University
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Abstract

The cocaine self-administration literature has provided considerable evidence for the central role of dopamine in the reinforcing effects of cocaine. It is also well established that dopamine is a modulator of electrical coupling via its effect on gap junctions. Gap junctions are formed of two membrane bound hemichannels which are composed of connexin proteins. Given that cocaine is a potent inhibitor of dopamine reuptake it is likely that cocaine would have an effect on gap junctions. These experiments examined whether cocaine self-administration in the rat would affect the brain expression of a gap junction channel forming protein named connexin 32. Using Western blot analysis, immunohistochemistry and Northern blot analysis; connexin 32 was examined following long term and acute exposure to cocaine, acute exposure to WF23 and long term exposure to heroin. Results demonstrated lasting changes in hippocampus and nucleus accumbens levels of connexin 32 protein and gene expression up to 21 days after the last injection of cocaine. Following acute exposure to cocaine, no changes in connexin 32 protein were visible. However, after a single injection of WF23 there were dramatic and long lasting changes in connexin 32 protein in the hippocampus, nucleus accumbens, striatum and thalamus. Long term heroin self-administration revealed a different pattern of connexin 32 protein changes which indicated that the previously seen patterns in Cx 32 expression were unique to cocaine and not general to any drug of abuse. In conclusion, these changes in connexin 32 expression may indicate a role for gap junction communication in the reinforcement processes and neuroadaptive changes produced by cocaine.

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Glossary

connexin: one of six transmembrane proteins that form a connexon

connexon: half a gap junction complex that is formed of six connexins

gap junction: a direct cytoplasmic connection between neighboring cells formed by the pairing of two connexons

hemichannel: synonym for connexon

heteromeric junction: a gap junction where each connexon is composed of two or more connexins

heterotypic junction: a gap junction where each connexon is formed of a different connexin

Northern blot: an electrophoresis technique which provides mRNA sizes and approximate concentration when transferred to membrane and labelled with a probe

probe: each gene has a unique nucleotide sequence so the mRNA can be detected with a labelled DNA fragment that is complementary to it called a probe

SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis

tubulin: an ubiquitous protein found in numerous cell types that is often used as a protein loading control for Western Blot analysis

Western blot: the SDS-PAGE of proteins which are then transferred to membrane and probed with antibodies to detect specific proteins

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The basic goal of drug self-administration research is to try and determine what are the underlying causes of addiction. Koob et al. (1998) best described it as "trying to understand what causes the shift between occasional drug use to the loss of behavioural control and drug-seeking that signal addiction". Changes in synaptic transmission in relation to cocaine self-administration have been extensively examined and a vast literature has developed that documents the importance of dopamine to the reinforcing effects of cocaine. The focus of this literature has been on the role of dopamine as a neurotransmitter. But dopamine is also a modulator of gap junctions in the central nervous system and this action has not been examined with respect to drugs of abuse. Cocaine, a dopamine reuptake inhibitor, would have effects on both dopaminergic neurotransmission and dopamine-modulated electrical coupling. This project examined the effect of cocaine self-administration on the gap junction protein, connexin 32.

1.0 Animal Models and Cocaine Self-administration Techniques

Animal models of drug self-administration have been used to examine the abuse liability of cocaine, patterns of cocaine intake and the neural substrates underlying cocaine reinforcement (Hubner & Koob, 1990; Loh & Roberts, 1990; Depoortere et al., 1993; Fitch & Roberts, 1993). In the sixties, techniques were developed that allowed laboratory animals to control the delivery of various drug solutions through an intravenous route (Weeks, 1961, 1962; Deneau et al., 1969). Both non-human primates and rats were shown to self-administer drugs such as morphine and cocaine. Drugs with a high abuse potential in the human population have since been shown to be self-administered by a variety of species. The pattern of self-administration demonstrated by these species is similar to the pattern of drug intake by human addicts. These data lend support to the

idea that the neural mechanisms responsible for drug reinforcement are fundamentally similar across species (Hubner & Koob, 1990; Depoortere et al., 1993; Fitch & Roberts, 1993). While no animal model fully captures the human condition of drug dependence, drug self-administration procedures provide useful methods to investigate the physiological and neurobiological mechanisms that mediate the reinforcing effects of drugs of abuse. The majority of cocaine self-administration studies in rats have used a simple fixed ratio schedule of reinforcement (Bozarth & Wise, 1985; Roberts & Ranaldi, 1995; Arnold & Roberts, 1997; Ahmed & Koob, 1998).

1.1 Role of Dopamine in Cocaine Reinforcement

Behavioural, pharmacological and molecular studies have established the importance of dopamine (DA) terminals, particularly within the mesolimbic DA system, in the reinforcing effects of cocaine (Roberts et al., 1989; Hubner & Moreton, 1991; McGregor & Roberts, 1993; Richardson et al., 1994; Smith et al., 1995).

Neurotoxic lesions have provided fundamental evidence for the role of DA in cocaine reinforcement. Cocaine blocks re-uptake at DA terminals and thereby acts as an indirect agonist at DA synapses (Hubner & Moreton, 1989; Britton et al., 1991). Therefore, the removal of the presynaptic terminal should eliminate cocaine's site of action. The regions with the highest DA content are the nucleus accumbens (ACC) and striatum (ST) which receive innervation from the ventral tegmental area and substantia nigra respectively. The relative importance of these dopaminergic innervations has been evaluated by ACC or ST lesions.

1.2 Cocaine and the Nucleus Accumbens

The importance of the dopaminergic role in the ACC is well illustrated by 6-hydroxydopamine (6-OHDA) lesions of the ACC. Bilateral infusions of the neurotoxin 6-OHDA into the ACC deplete DA levels and disrupt cocaine self-administration. Partial depletion of the ACC produces a partial disruption in cocaine intake. This procedure induces either post-lesion extinction behaviour or a complete loss of cocaine self-administration behaviour if DA depletion is greater than 80%. This indicates a critical role for the dopaminergic innervation of the ACC in cocaine reinforcing mechanisms (Roberts et al., 1977;1980).

The ACC appears to be the central brain region responsible for the reinforcing properties of cocaine and the site of chronic adaptation to drug reinforcement (Nestler, 1994; Peoples et al., 1997). The dopaminergic projections of the ventral tegmental area to the ACC are considered pivotal to many aspects of "reward" including cocaine self-administration (DiChiara & Imperato, 1988; McGregor & Roberts, 1993; Nestler, 1996). There is a significantly increased level of DA in both the ACC and prefrontal cortex following cocaine self-administration (Zeigler et al., 1991; Koob, 1992). Electrophysiological recordings from the ACC demonstrate several consistent patterns of responding to cocaine self-administration. Increased levels of extracellular dopamine in the ACC are detected through microdialysis in rats that are self-administering cocaine (Hurd et al., 1989; Pettit & Justice, 1990; 1991; Robledo et al., 1992; Weiss et al., 1992). The ACC is also the primary region where chronic cocaine self-administration causes a decrease in D₁ receptor density (Graziella De Montis et al., 1998). The ACC is extremely sensitive to direct injection of D₁ or D₂ receptor antagonists which produce increases in the rate of cocaine self-administration

(Britton et al., 1991; Corrigan et al., 1991; Robledo et al., 1992; Caine et al., 1995; Koob & LeMoal, 1997).

1.3 Cocaine and the Striatum

While the dopaminergic activation of the ST, specifically D_1 receptor activation, has been implicated in some behavioural actions of cocaine (Thomas et al., 1996), the ST does not seem to be essential to the reinforcing effects (Roberts & Zito, 1987; Glick et al., 1988). Cocaine induces behavioural sensitization such as increased locomotor activity and stereotypy which is associated with dopaminergic nigrostriatal projections and striatal activation (Kennedy & Hanbauer, 1983; Bowman & Kuhn, 1996; Pederson et al., 1997). The ST is the definitive site for the chronic changes in the level of DA that underlie the behavioural activation of cocaine (Martin-Fardon et al., 1997; Little et al., 1998). This behavioural activation is often used to confirm the effectiveness of cocaine analogues (Cline et al., 1992; Porrino et al., 1994; Porrino et al., 1995; Fleckenstein et al., 1996).

1.4 The Dopamine Transporter

Cocaine causes elevated extracellular DA in the ACC and ST by inhibiting the re-uptake of DA by the dopamine transporter (Martin-Fardon et al., 1997; Little et al., 1998). The dopamine transporter mediates the uptake of DA into neurons by an electrogenic Na^+/Cl^- transport mechanism (Reith et al., 1997). The transporter clears DA from the synaptic cleft and returns it to the presynaptic terminals. Cocaine blocks the dopamine transporter which leads to increased DA in the extracellular space which then causes a potentiation of DA transmission (Carroll et al., 1992; Cerruti et al. 1994). The dopamine transporter has been sequenced and cloned and now

there is a great effort to try and find an analogue that will bind to the transporter and prevent the actions of cocaine without disrupting DA uptake (Hubner & Moreton, 1991; Rothman, 1991; Shimada et al., 1992; Roberts, 1993; Roberts & Rinaldi, 1995).

1.5 Cocaine Research and Molecular Biology

Molecular biology is starting to make enormous contributions to the cocaine literature by demonstrating how, through the alteration of protein synthesis and gene transcription, neurotransmitters change the types of receptors and the intracellular functionality of the target neurons (Nestler, 1994). These studies have suggested a more intricate basis for cocaine reinforcement and dependence beyond the DA hypothesis. The development of cocaine biochemical probes in combination with imaging techniques greatly increased the focus on both DA and other transporters. These techniques provide information on cocaine binding site location, density, and characteristics since the probes are nearly identical to cocaine (Fowler et al., 1989; Carroll et al., 1992; Meltzer et al., 1993). At an even more basic level DA can effect gene expression which then effects protein transcription. Overall, the implication of these studies is that cocaine exerts its effect on the neuron far beyond the initial interaction with re-uptake proteins.

A dopamine transporter knockout is the most effective way to delineate the importance of this protein to cocaine reinforcement. Giros et al. (1996) used a mouse strain in which the gene for the dopamine transporter was knocked out. Consequently, these mice also had fewer DA receptors, decreased DA neuronal stores, and increased extracellular DA (causing hyperactivity). Even without the dopamine transporter these mice could still be trained to self-administer cocaine although it should be emphasized that these mice already had high levels of extracellular DA and

there was delayed acquisition (Caine, 1998; Rocha et al., 1998). Cocaine binding sites were mapped in these dopamine transporter knockout mice and results showed binding in the ACC, hippocampus, thalamus and to a lesser degree the ST (Rocha et al., 1998).

1.6 G Proteins and Cocaine

In order for DA to have any kind of effect on a target neuron there is a cascade of intracellular messengers such as G_i , G_o , cAMP and Ca^{2+} (Nestler, 1994). These messengers will also affect protein phosphorylation which alters the function of numerous neuronal proteins. Chronic D_1 receptor stimulation has been shown to decrease levels of G_i in the ACC. In the ST D_1 stimulation leads to activation of G_i proteins and increased intracellular cAMP (Self et al., 1994; Hyman et al., 1996; Moratalla et al., 1996). This increase in cAMP may lead to immediate-early gene induction (*c-fos*) and the phosphorylation of CREB [cAMP response element-binding protein] (Harlan & Garcia, 1998). This cycle appears to favour chronic cocaine self-administration given that D_1 receptor antagonists have anti-cocaine properties. Also implicated in the effects of cocaine is the D_2 receptor (present both pre and post synaptically) which activates G_i proteins (Reith et al., 1997). In the ventral tegmental area, chronic cocaine self-administration causes decreases in G_i proteins which leads to D_2 receptor subsensitivity (Nestler, 1994).

1.7 Cocaine's Activation Beyond the Nucleus Accumbens and Striatum

Cocaine's activation of dopaminergic circuits extends beyond the ACC and ST (Bardo, 1998). Chronic cocaine exposure increases the turnover rate of DA in the hippocampus (HIP) and leads to the development of behavioural sensitization (Dworkin et al., 1995). The distribution of cocaine during behavioural sensitization has been examined and the highest levels of cocaine were

found in the ST, HIP, and thalamus (Thomas et al., 1996; Bonate et al., 1997; Breiter et al., 1997). CA1 neurons of the HIP exhibit increased spontaneous firing in the presence of both DA and cocaine suggesting a role in seizure epileptogenesis (Stein & Belluzzi, 1989; Change et al., 1998). Cocaine induced seizures are caused by the blockade of DA re-uptake and the subsequent elevation of excitatory agonists. Zhai et al., (1997) demonstrated that chronic low dose exposure to cocaine increases the likelihood of seizure activity in response to low doses of a convulsant drug. This reduction in drug threshold was due to the increased synchronized bursting of hippocampal CA1 neurons. Preliminary research indicates that this increased synchronous firing is DA modulated (Perez Velazquez et al., 1997). Hippocampal G proteins are affected by cocaine administration. Chronic and acute cocaine decreased the level of G_i mRNA in the CA3 and increased the level in the CA1 at several times. All subfields of the HIP have shown time course dependent changes in G protein expression following exposure to cocaine (Przewlocka et al., 1994).

The thalamus (TH) is another region implicated in the behavioural sensitization observed following exposure to cocaine (Biegon et al., 1992; Thomas et al., 1996; Bonate et al., 1997; Breiter et al., 1997). DA may modulate the excitability of thalamic cells (Lavin & Grace, 1998). It has also been speculated that the TH may mediate the analgesic effects of cocaine (Dougherty et al., 1990; Shyu et al., 1992). Lesions of several thalamic nuclei have provided a variety of results. For example, rats with lesions of the mediodorsal nucleus self-administered significantly less cocaine than controls (Weissenborn et al., 1998). Lesions of the paraventricular nucleus enhanced the locomotor response to acute cocaine injection whereas the behavioural sensitization from

chronic cocaine was inhibited (Young & Deutch, 1998). In the thalamus, cocaine causes significant changes in dopaminergic and serotonergic function as demonstrated by an increased DA level, an increased serotonin level, and a decreased 5-hydroxyindole acetic acid/serotonin ratio (Hadfield & Milio, 1992; Hadfield, 1995).

1.8 Cocaine and Norepinephrine

Norepinephrine has been shown to interact with DA in the ACC and this interaction may lead to the behavioural sensitization which occurs following repeated administration of cocaine (Cools, 1991). Cocaine has its effect on norepinephrine neurons by preventing re-uptake by binding to the norepinephrine transporter. Evidence supporting the involvement of norepinephrine has been mixed (Tella, 1995; Mello & Negus, 1996; Kleven & Koek, 1998). Lesion of the noradrenergic system does not affect cocaine self-administration (Roberts et al., 1980; Roberts et al., 1994). DA uptake blockers like GBR 12935 and GBR 12909 increase DA and norepinephrine release in the ventral tegmental area (Reith et al., 1997). Use of the norepinephrine selective re-uptake inhibitor desipramine has produced small but significant decreases in cocaine self-administration (Tella, 1995). Cocaine has been shown to increase extracellular norepinephrine in the prefrontal cortex (part of the mesolimbic dopaminergic system implicated in the reinforcing effects of cocaine) in a similar quantity to DA (Tanda et al., 1997). In fact, cocaine may require norepinephrine to cause the dopaminergic increase seen in the prefrontal cortex. Tanda et al. (1997) demonstrated that introduction of desipramine (norepinephrine carrier blocker) into the prefrontal cortex prevented cocaine from increasing DA levels in the prefrontal cortex.

1.9 Cocaine and Serotonin

Similar to norepinephrine, cocaine prevents the re-uptake of serotonin through transporter binding and serotonin uptake inhibitors have been shown to modulate the reinforcing effects of cocaine (Peltier & Schenk, 1993; Satel et al., 1995; Cunningham et al., 1996). Chronic cocaine exposure decreases serotonin turnover and decreases the effect of serotonin on dopaminergic activity (Dworkin et al., 1995; Norton & Galloway, 1996). Kleven & Koek (1998) found that cocaine's behavioural and biochemical effects were due mainly to inhibition of the dopamine transporter and serotonin transporter. Lesions of the medial forebrain bundle with the serotonin toxin 5,7-dihydroxytryptamine increased the reinforcing efficacy of cocaine (Loh & Roberts, 1990). Pretreatment with fluoxetine (an indirect serotonin agonist) and L-tryptophan (increases serotonin) decreased the reinforcing efficacy of cocaine (Richardson & Roberts, 1991; McGregor & Roberts, 1993). Recent advances in molecular techniques have also shown cocaine withdrawal related increases in immediate-early gene expression in both the dopaminergic and serotonergic regions of the brain (Harlan & Garcia, 1998; Humblot et al., 1998; Rocha et al., 1998). This suggests that serotonin may modulate the craving and withdrawal associated with chronic cocaine exposure (Aronson et al., 1995; Satel et al., 1995; Burchett & Bannon, 1997). Parsons et al. (1996) reported decreased extracellular serotonin in the ACC of rats going through cocaine withdrawal and the significance of the decrease was contingent on the length of time cocaine was self-administered.

1.10 Dopaminergic Changes During Cocaine Withdrawal

There are major and long-lasting changes in the mesocorticolimbic system following withdrawal from chronic cocaine self-administration (Koob et al., 1997; Zhang et al., 1998). Extracellular levels of DA fluctuate during withdrawal and there are decreases in ACC and prefrontal cortex DA receptive neurons and transmission (Kuhar & Pilotte, 1996). Immediately following withdrawal and up to four days later there is increased mesolimbic extracellular DA (Weiss et al., 1992). Dopamine transporters also show changes following cocaine withdrawal (Kuhar & Pilotte, 1996). Pilotte et al. (1994) demonstrated an increase in dopamine transporters in the ACC three days after last exposure to chronic cocaine which then dropped to 1/3 the baseline amount one week later. Reduction in dopamine transporters persisted up to two months. These changes in dopamine transporter level were likely due to the concurrent decrease in transporter mRNA which suggests cocaine withdrawal has a direct effect at the gene expression level (Cerruti et al., 1994). The significance of these mesolimbic changes during withdrawal relates directly back to the role of the ACC in drug craving (Zhang et al., 1998). Ahmed & Koob (1998) demonstrated that rats allowed long periods of access to cocaine would show, following withdrawal and subsequent re-exposure, a dramatic increase in cocaine intake. If the ACC is integrally related to cocaine reinforcement then the DA and dopamine transporter irregularities within this region may underlie what we define as drug craving during withdrawal.

Human cocaine withdrawal studies have demonstrated regions of DA uptake and shown that during cocaine withdrawal there is a decrease in prefrontal cortex blood flow and a decrease in glucose metabolism in the basal ganglia for up to three months (Pike, 1993; Volkow et al.,

1992, 1993). Additional studies have shown increased metabolic activity in the hippocampus and nucleus accumbens which correlated with cocaine cravings in cocaine-dependent subjects (Breiter et al., 1997). Human brain imaging studies using PET have shown definitive changes in brain circuitry before and after repeated exposure to cocaine, little change in the availability of dopamine transporters and an overall decrease in metabolic brain activity (Volkow et al., 1993; 1996; 1997). Chronic exposure to cocaine definitely produces long-lasting adaptations in brain neurochemistry and in systems associated with drug reinforcement.

1.11 Acute Versus Chronic Cocaine Self-administration

Acute and chronic cocaine self-administration produce different physiological effects. Acute cocaine increases both heart rate and blood pressure dramatically but tolerance quickly develops; chronic cocaine exposure has little continuing effect on heart rate or blood pressure (Ambrosio et al., 1996). Chronic cocaine intake has documented effects on the immune system, including altered T cell and macrophage function, whereas acute cocaine has no established immune changes (Baldwin et al., 1998). Acute exposure to cocaine induces the immediate early gene Fos in the ACC (Graybiel et al., 1990; Cole et al., 1992). Chronic exposure to cocaine also induces immediate early gene expression of Fos-like proteins but they are different from the acute Fos expression. In fact, Hope et al. (1994) found that chronic Fos proteins have much longer half lives than acute Fos (12-24 hrs) and that up to one week after withdrawal from cocaine levels of chronic Fos were still elevated. Nestler (1994) best described the significance of these results as the first data demonstrating not only a quantitative difference but also a qualitative difference between chronic and acute cocaine self-administration. Other chronic adaptations include: an

increase in ventral tegmental area tyrosine hydroxylase, a decrease in phosphorylated ACC tyrosine hydroxylase, and a decrease in ACC G_i protein (Beitner-Johnson & Nestler, 1991; Terwilliger et al., 1991; Nestler et al., 1993; Self & Nestler, 1995).

1.12 Cocaine Analogues: WF23

Cocaine self-administration research has expanded to include a variety of analogues. The potency of a given cocaine analogue in sustaining self-administration is predicted by the analogue's affinity for the dopamine transporter (Kennedy & Hanbauer, 1983, Carroll et al., 1992; Meltzer et al., 1993). In theory, a cocaine analogue with high affinity to the DA transporter, a low dissociation rate and low non-specific binding may be useful in the treatment of cocaine addiction. Cocaine and dopamine bind to different recognition sites on the dopamine transporter (Kennedy & Hanbauer, 1983; Madras et al., 1989). The behavioural effects of cocaine are related to the potency of cocaine at the dopamine transporter binding site. A drug that is more potent than cocaine binding to the transporter should produce stronger behavioural effects (Cline et al., 1992; Porrino et al., 1994; Porrino et al., 1995). Davies et al. (1993) developed a new group of cocaine analogues called tropanes which are based on the reaction of vinylcarbenoids with pyrroles rather than using cocaine as the building block. These new analogues allow for a more detailed structure-activity analysis of cocaine at transporter sites. These compounds can be used to examine the specific effect of cocaine on the dopamine transporter such as physical blockage of DA re-uptake versus modulation of the transporter (Boja et al., 1994). Several analogues have been examined for their ability to support self-administered behaviour with mixed results, but

most have demonstrated a higher potency than cocaine (Weed & Woolverton, 1995; Nader et al., 1997).

WF23 is long acting cocaine analogue that has high and non-selective affinity at the DA, norepinephrine and serotonin transporter and is readily self-administered by rats (Roberts et al., in press). WF23 is 230 times more potent than cocaine at inhibiting DA uptake, 1500 times more potent than cocaine at inhibiting serotonin uptake and 270 times more potent than cocaine at inhibiting norepinephrine uptake (Bennett et al., 1995). Both cocaine ester groups have been removed from the WF23 analogue which makes it more metabolically stable since 80% of cocaine's metabolism occurs through hydrolysis of the ester groups (Hemby et al., 1995). This makes WF23 an ideal candidate for psychostimulant studies when cocaine is not a suitable compound.

1.13 Other Drugs of Abuse: Heroin

Heroin is a highly addictive drug and that has established synergistic DA effects when used in combination with cocaine (Azaryan et al., 1998; Herz, 1998; Hemby et al., 1999). There are many biological crossovers between cocaine and heroin though ultimately their mechanisms of action are separate. Heroin self-administration has been examined with regards to the mesolimbic DA system (Koob, 1992). There is an indirect increase (through GABAergic inhibition) in dopaminergic transmission in the ACC following direct injection into the ventral tegmental area (Bozarth & Wise, 1981; Devine & Wise, 1994). Results have shown that the level of DA released in the ACC following heroin was comparable to what would be expected following cocaine self-administration (Wise et al., 1995). Opiate receptors are found primarily in the ventral

tegmental area in proximity to the dopaminergic cell bodies (Devine & Wise, 1994). These receptors inhibit adenylyl cyclase through a pertussis toxin-sensitive G_i protein (Nestler, 1992; Guitart & Nestler, 1993). DA lesions of the ACC do not prevent heroin self-administration whereas DA depletion in the ventral tegmental area decreases heroin intake (Pettit et al., 1984; Bozarth, 1987). Heroin will be directly self-administered into the ACC and potentially in the HIP as well (Vaccarino et al., 1985; Stevens et al., 1991). Heroin is a drug of abuse that has distinct reinforcing and withdrawal effects in comparison to cocaine.

1.14 Cocaine: Future Directions

Cocaine research is directed towards finding what causes that shift from occasional drug use to the loss of behavioural control and drug-seeking that signal addiction. The majority of cocaine research still continues to focus on neurochemical changes within specific brain regions or changes related to the dopamine transporter. Molecular biology is starting to make headway into the neurobiology underlying cocaine addiction by examining how cocaine alters protein synthesis, gene transcription, and the intracellular functionality of the target neurons. Molecular technology could also be applied to examine the effect of cocaine on electrical coupling. Electrical coupling through gap junctions is a fundamental means of communication between cells that remains unexplored in the cocaine literature.

2.0 Gap Junction Structure

A gap junction is formed by two hemi-channels called connexons imparted by each of the coupled cells and these gap junctions aggregate in the plane of a membrane to form gap junction plaques (Kumar & Gilula, 1992, 1996; Perkins et al., 1997). Plaques are regions where the

repulsive force between membranes is minimized and these regions are dense with connexons in order to minimize the amount of energy required to maintain the channel. There is a very slight 2 nm gap between the two opposing cell membranes. The opening and closing of the hemichannel is regulated from the cytoplasmic side and the communication with other hemi-channels is regulated from the extracytoplasmic side (Dermietzel et al., 1990). These hemi-channels, with an approximate width of 1.2 nm, allow passage of molecules up to one kilodalton in size which may have a positive or negative charge or be neutral (Bennett et al., 1991).

The aqueous pore formed by the two hemi-channels provides direct selectively permeable cytoplasmic contact between two different cells (Dermietzel et al., 1990). The pore of the connexon is formed by the polar side of the amphipathic helix of the third transmembrane domain (M3) of each respective connexin which is a protein that forms a connexon (Sosinsky, 1996). Across all connexins, M3 has conserved polar, acidic and basic regions that are separated by three residues (Bennett et al., 1991). Molecules such as Ca^{2+} , IP_3 , cAMP, amino acids and vitamins can pass without allowing any proteins or nucleic acids (Bevans et al., 1998). The connexon hemi-channels are regulated so that they are closed under normal conditions (Cascio et al., 1995; Rhee et al., 1996). The conductance of a single gap junction channel is no larger than a simple ligand gated channel even though it has a much larger pore size and increased permeability to ions. The decreased conductance is undoubtedly due to the length of the channel and the fact that it must span two membrane bilayers rather than one (Unwin, 1989; Harris et al., 1992).

Factors that affect the regulation of a gap junction also determine the function and permeability of the pore. Gap junction channel conductance can be changed through

manipulations in Ca^{2+} level, pH, and second messengers (Spray & Bennett, 1985; DeVries & Schwartz, 1989; Traub et al., 1989; Dermietzel et al., 1990; Saez et al., 1990; Kojima et al., 1996). Gap junctions have differing functional capacities depending on the type of connexin forming the hemi-channel (Bevans et al., 1998). The connexins that form the pore ultimately determine the size and charge transfer of molecules allowed to pass through the pore, have different voltage sensitivities, have different responses to phosphorylating agents and selectively propagate different second messenger molecules between cells (Bennett et al., 1991; Giaume et al., 1991; Dermietzel & Spray, 1993; Paul, 1995; Bruzzone et al., 1996b).

2.1 The Connexin Family

Connexons are encoded by a gene family with two separate lineages. The most basic family division consists of the beta family which includes connexin 32 and connexin 26 and the alpha family which includes connexin 43 (Dermietzel & Spray, 1993). Hydropathy analysis determines the differences between the two families in that alpha members have a larger cytoplasmic C terminal and a larger cytoplasmic loop between second and third transmembrane domains (Yeager & Nicholson, 1996). Each connexon is formed of six proteins called connexins and to date there are 13 distinct mammalian connexins (summarized in Table 1) that have been characterized, each encoded by a single copy gene (Paul, 1995; Bruzzone et al., 1996a). The connexins were named according to their apparent molecular weight position determined by SDS-PAGE (Paul, 1986).

Connexins are part of the four transmembrane (M1-M4) domain family (see Figure 1).

Connexins are grouped together at the level of the phospholipid bilayers and extend the length of

Table 1. Summary of all cloned connexins and their predominant location

Cloned Connexin	Region of the Highest Connexin Density
Cx 26	hepatocytes, pinealocytes, pancreas, endometrium
Cx 30	brain, skin
Cx 30.3	skin
Cx 31	placenta, keratinocytes
Cx 32	hepatocytes, neurons, oligodendrocytes, Schwann cells
Cx 33	testes
Cx 37	lung, endothelium, cortical neuroblasts
Cx 40	lung, endothelium, smooth muscle
Cx 43	astrocytes, cardiac muscle, smooth muscle, fibroblasts
Cx 45	kidney, skin, lung, embryonic brain
Cx 46	lens fiber cells
C 50	lens fiber cells, corneal epithelium

Figure 1. Adaptation of a generic connexin from Bruzzone et al., 1997. M1-M4 represents the four transmembrane domains, E1 and E2 are the two extracellular loops, CL the cytoplasmic loop, NT the amino terminus and CT the carboxy terminus.

the plasma membrane (Goodenough et al., 1988; Miller et al., 1988; Kumar & Gilula, 1996a). These four domains (of 20-28 amino acids each) are hydrophobic and retain 90% homology across connexins (Dermietzel et al., 1990). Both the N and C termini are cytoplasmic with one cytoplasmic loop (between M2 and M3) and two extracellular loops (Hertzberg et al., 1988; Dermietzel & Spray, 1993). The N terminal is roughly 50% conserved across connexins. The conserved sequences may act as leader sequences for membrane insertion since connexins have no signal sequence (Paul, 1995; Bruzzone et al., 1996a). The extracellular loops (of about 40 amino acids each) are cysteine rich and have a high degree of homology due to the common function of extending across the gap, insulating the channel and docking with other connexons (Bennett et al., 1991; Sosinsky, 1996; Foote et al., 1998). The C terminus and cytoplasmic loop are the least conserved across connexins and may account for the unique physiological properties of a specific connexin channel and the differential regulation of the hemi-channel through presence or absence of phosphorylation sites (Sosinsky, 1996; Yeager & Nicholson, 1996).

2.2 Gap Junctions and Development

Gap junction coupling in the developing brain may be creating the blueprints for synapse and cortical circuit formation in the adult brain (Kandler & Katz, 1995; Bruzzone et al., 1996b). Gap junctions play a vital role in the intercellular communication between cells during the early stages of embryogenesis and into the final stages of development when junctions form compartmentalized communication domains (Hertzberg et al., 1980; Fulton, 1995; Kandler & Katz, 1995; Paul, 1995; Bhat, 1998). This differentiation in coupling is easily visualized as changes in dye-transfer pattern between cells (Guthrie & Gilula, 1989). Electrical coupling is

responsible for the coordination of physiological systems within an organ such as the rhythmic contraction of the heart or buffering the K^+ activity around neurons (Guthrie & Gilula, 1989; Jefferys, 1995). Gap junctions also organize cells into tissue, coordinate cellular function and delineate cell populations within the brain which function as interacting compartments (Dermietzel & Spray, 1993; Bhat, 1998). The pattern of connexin expression in the brain changes during stages of development (Belliveau et al., 1991). There is an established relationship between connexin 26 and connexin 32 during brain development. During embryonic development there is a predominance of connexin 26 with almost no connexin 32 followed by a subsequent decrease to almost zero expression of connexin 26 a month after parturition (Dermietzel & Spray, 1993). Cx 32 has the reverse cycle showing a large increase just as connexin 26 declines (Dermietzel et al., 1989; Dermietzel et al., 1990). Peinado et al. (1993) studied neocortical neuronal gap junctions at different stages of development through dye coupling. At early postnatal stages coupling was heavy between neurons at dendrites whereas at later time points coupling was drastically decreased suggesting that gap junctions are necessary during early development to coordinate circuit formation and guide synaptic establishment. As development progresses, areas of gap junction coupling remain widespread but the connexin proteins forming the hemichannels become restricted to discrete areas.

2.3 Connexin Distribution in the Brain

Connexin expression overlaps across cell types but each connexin has its own regional pattern of expression (Goodenough et al., 1988; Kumar & Gilula 1996a). Connexins 26, 43 and 32 are the major connexins expressed in mammalian CNS gap junctions. Within the rat cortex

approximately 72.5% of gap junctions are between astrocytic processes, 17.5% between astrocytes and a neural process, 6.5% astrocyte - oligodendrocyte and 2% neuronal-neuronal (Nadarajah et al., 1996). Connexin 26 has its highest level of expression during development and then becomes restricted to mostly non-neuronal cells. Connexin 43 is the most abundant of the connexins and is found in astrocytes and neurons of the cerebellum, occipital cortex, neocortex and HIP (Hertzberg & Skibbens, 1984; Dermietzel et al., 1989; Micevych & Abelson, 1991; Nadarajah et al., 1996; Simburge et al., 1997). Connexin 32 (Cx 32), found mostly in liver and kidney, is expressed by both neurons and oligodendrocytes (Hertzberg & Skibbens, 1984; Paul, 1986; Dahl et al., 1987; Dermietzel et al., 1989; Naus et al., 1990; Belliveau et al., 1991, Yamamoto et al., 1991; Bennett, 1994; Scherer et al., 1995; Kumar & Gilula, 1996b; Nadarajah et al., 1996; Dermietzel et al., 1997; Li et al., 1997).

The use of Cx 32 cDNA has demonstrated the presence of Cx 32 mRNA in the pons, cerebellum, medulla, olfactory bulb, midbrain, striatum, thalamus, amygdala, occipital cortex, frontal cortex and hippocampus (Dermietzel et al., 1989; Shiosaka et al., 1989; Yamamoto et al., 1989; Naus et al., 1990; Micevych & Abelson, 1991; Belliveau & Naus, 1995). The levels varied greatly between regions with the hindbrain having the highest levels and the cortices and amygdala the lowest. Immunohistochemistry has also been used to examine the distribution of Cx 32 immunoreactivity within the hippocampus. The highest level of Cx 32 protein was found in the pyramidal cell layer and at the border of the molecular and granule cell layers in the dentate (Yamamoto et al., 1989). There was also some immunoreactivity within the neuronal perikarya in the CA2 and CA3 areas.

2.4 Connexin Gene Structure

The structure of connexin genes is similar across all known connexins (Hoh et al., 1991; Sohl et al., 1996). The reading frame is uninterrupted and located in the second exon which is separated from the 5' untranslated end (exon 1) by a variable length single intron (Miller et al., 1988; Bennett et al., 1991; Bruzzone et al., 1996b). The length of the intron varies by connexin with a range of approximately 3.8-10.5 kb (Sohl et al., 1996). The Cx 32 exons are separated by a 6.1 kb intron. Cx 32 promoters (in rat) are not entirely characterized but it is known that the Cx 32 gene is alternatively spliced by three different promoters depending on the cell type (Hennemann et al., 1992; Neuhaus et al., 1995; Sohl et al., 1996). The gene for Cx 32 is located on chromosome X (Ionasescu et al., 1996a, 1996b).

2.5 Connexin Trafficking

Trafficking of the individual connexins within the cell has yet to be clearly demonstrated. Connexin immunoreactivity has been localized to the cytoplasm of many cell types (Swenson et al., 1989; Yamamoto et al., 1989; Yamamoto et al., 1990; Bond et al., 1992; Bond et al., 1994). The first step in connexon assembly is the oligomerization of six connexins. Connexins are cotranslationally inserted into the endoplasmic reticulum and are probably oligomerized in the trans-golgi network (Musil & Goodenough, 1993; Bruzzone et al., 1996b). Connexins are likely available in a pool and transported within a vesicle which is easily moved by a trans-golgi network transportation system (Musil & Goodenough, 1993). In support of this, the short latency with which coupling develops and the immediate lack of effect of protein inhibitors appears to indicate that there is a pool of available connexin protein (Bennett et al., 1991). The rapid turnover of

connexins makes them different from other integral membrane proteins. It is not clear if connexin proteins associate into connexons in the cytoplasm or in the cellular membrane. The high level of connexins present in the golgi apparatus could indicate a site of oligomerization which would suggest that fully formed connexons would be present in the cytoplasm travelling to the cellular membrane (Musil et al., 1990; Musil & Goodenough, 1993).

2.6 Connexin Docking

Connexin docking and the stability of the channel are integral to the structure of the intercellular channel. Gap junctions are known to associate in tightly packed areas called plaques which minimizes the repulsive forces between cells (Sosinsky, 1996). The density and stability of the plaque requires the presence of lipids and cholesterol between the channels. Cell surface glycoproteins and adhesion molecules also have an established role in the forming of gap junctions and aid in the exchange of information between cells and the extracellular matrix (Musil et al., 1990; Wolburg & Rohlmann, 1995; Prowse et al., 1997). Liver cell adhesion molecules (L-CAM) increase gap junction coupling and anti-L-CAM antibodies disrupt coupling. Mege et al. (1988) transfected L-CAM into cells that were previously deficient in adhesion molecules and were incompetent for gap junction communication. Following L-CAM transfection, functional gap junctions appeared. Ductin, a 16-kDa protein that is often co-isolated with gap junctions, has also been suggested as a possible constituent protein of a formed gap junction (Finbow & Pitts, 1993; Finbow et al., 1995; Wolburg & Rohlmann, 1995). The binding between the two hemichannels appears to be by noncovalent hydrogen bonds which stabilize the alignment of the channel across

the gap (Manjunath & Page, 1985). The strength of the bonds holding the hemichannels varies from tissue to tissue.

2.7 Gap Junction Gating

Gap junction physiology demonstrates a complex process of control over the gating of the intercellular channel. Gap junctions can quickly close down conductance and then as rapidly reverse to an open state leading to the predominant hypothesis that the channel functions on an "all or nothing" principle (Bennett et al., 1991; Bruzzone et al., 1996a). Gap junction channels are responsive to a variety of treatments. Treatment with high Ca^{2+} concentration solutions appears to close the channel (Spray et al., 1985; Unwin, 1989). Channels also close in response to lipophilic molecules like halothane and octanol which act by incorporating into the lipid bilayer (Nedergaard et al., 1995). Phosphorylation of connexins is often correlated with increased conductance due to activation of cAMP and protein kinase C (Saez et al., 1990). These immediate effects also regulate further down the cascade in that cAMP once activated is thought to have effects on connexin transcription and mRNA stability (Spray et al., 1987; Bevens & Harris, 1999). This can have an effect on an entire cell circuit since second messengers can diffuse through the gap junction channel and have continued post-synaptic effects (Dermietzel & Spray, 1993). These signalling molecules have short half lives so even a slight change in hemichannel permeability can have effects on intercellular signalling transduction (Paul, 1995).

Results on the gating sensitivity of Cx 32 are mixed. Cx 32 gating sensitivity to Ca^{2+} is determined by the interaction of the C terminal and the cytoplasmic loop (Boitano et al., 1998; Wang & Peracchia, 1998a). Protein kinase C has been shown to cause minor Cx 32

phosphorylation at the Ser-233 site (Saez et al., 1986, 1990; Takeda et al., 1990). An elevation of cellular cAMP increases gap junction conductance in liver cells composed mainly of Cx 32 (Saez et al., 1986). cAMP response elements are also close to the transcription site for the Cx 32 gene which may lead to increased Cx 32 transcription (Miller et al., 1988). Interestingly, prior to membrane docking, cAMP can inhibit homomeric Cx32 channels and heteromeric Cx32/Cx26 channels prior to their complete incorporation into the membrane (Bevans & Harris; 1999). It should be noted that Cx 32 phosphorylation has not been clearly associated with changes in gap junction coupling. Channel gating really depends on the connexins that are forming the connexon and whether the connexons are homotypic or heterotypic. For instance, homomeric Cx 32 channels are permeable to both cAMP and cGMP whereas Cx 32/Cx 26 heteromeric channels are permeable only to cGMP due to the narrowed pore size (Bevans et al., 1998) The gating benefit of multiple connexins is the potential diversity in response to a second messenger which then influences the cellular response (Paul, 1995).

2.8 Connexin Degradation

The degradation of a gap junction is a three step process of dissociation, internalization and proteolysis. Gap junctions are very resistant to dissociation and the cycle of the connexon ends when the entire gap junction complex is internalized by one of the cells and is subsequently degraded by lysosomal or proteasomal proteolysis (Goodenough, 1988; Beardslee et al., 1998). This is an actin-dependent contractile mechanism that may be associated with phosphorylation of connexin amino acids (Swenson et al., 1990). It has also been suggested that outside the plaque connexons may be available or may exist in a semi-degraded state but are anchored in the

cytoskeleton and are therefore unusable (Wolburg & Rohlmann, 1995). The apparent half-life of Cx 32 is five hours (Fallon & Goodenough, 1981). This is an extremely short half-life in comparison to other membrane proteins which range from 23-78 hours (Fallon & Goodenough, 1981). Other connexins will have varying half-lives due to the differences in amino acid sequences and regulatory properties. For instance, Cx 43 has a half-life of 1.5-2 hours in both neonatal and adult heart cells (Laird et al., 1991; Beardslee et al., 1998). The short half-life of Cx 32 suggests that a block in protein synthesis which depletes the connexin pool would result in a rapid decrease in *de novo* connexon assembly. This would regulate intercellular communication and make the cells more responsive to physiological changes (Traub et al., 1989). Therefore intercellular communication may involve not only molecule passage but also changes in connexin protein synthesis, transportation, docking or degradation (Bruzzone et al., 1996b).

2.9 Heterotypic and Heteromeric Junctions

The presence of multiple connexins within the same cell suggests that some cellular functions may require hemichannels composed of more than one connexin with each connexin forming channels with unique molecular size and ion permeability (Willecke & Haubrich, 1996). A single cell expressing multiple connexins could potentially form both heterotypic (each connexon composed of a different connexin) and heteromeric (each connexon composed of two or more connexins) hemi-channels (Traub et al., 1989; Bennett et al., 1991; Dermietzel & Spray, 1990; Bennett, 1994; Bruzzone et al., 1996b; Wang & Peracchia, 1998b). The concept of heterotypic coupling depends on the conserved areas of the extracellular loops across connexins such that different connexins will recognize each other and form functional channels (Yeager & Nicholson,

1996). RNA-injected oocytes will form functional homomeric channels of Cx32/Cx32, Cx43/Cx43 and heterotypic channels of Cx32/Cx43 (Swenson et al., 1989). Heterotypic and heteromeric gap junctions have different gating properties because the voltage dependent gating of a hemichannel is dependent on the type of connexin forming the partner hemichannel (Swenson et al., 1989; Willecke & Haubrich, 1996; Wang & Peracchia, 1998b). These differences between hemichannel electrical and gating properties are easily illustrated by dye coupling (Robinson et al., 1993; Zahs et al., 1998). For example, biotin will pass from astrocytes to oligodendrocytes and astrocytes and Muller cells but not in the opposite direction. Dye-coupling has also demonstrated that Cx 32 and Cx 26 are capable of forming heteromeric gap junctions (Stauffer, 1995; Lee & Rhee, 1998). To date, the cell specific distribution of the connexin proteins or the composition of the hemi-channel have not been linked to a target function.

2.10 Functional Single Hemichannel

There is the possibility that a single hemichannel could operate independently of a gap junction complex. Hemichannels are present in the membrane prior to joining a connexon from a neighbouring cell (Bennett et al., 1991; Musil & Goodenough, 1993). There are electrophysiological demonstrations of unpaired Cx 46 connexons forming a single transmembrane channel with no coupling under stringent conditions (Goodenough et al., 1990; Paul et al., 1991). DeVries & Schwartz (1992) reported the appearance of uncoupled connexons in retinal cone horizontal cells but the connexin makeup was unknown. Other groups have established that Cx 32 when applied to single lipid bilayers will form single membrane channels (Harris et al., 1992; Rhee et al., 1996). These single Cx 32 connexons share common features

with gap junctions in that they are permeable to sucrose and Lucifer Yellow, reversibly reduced by acidic pH, and show asymmetric voltage sensitivity (Harris et al., 1992; Rhee et al., 1996). It appears that the single hemichannel is closed under normal conditions.

2.11 Gap Junction Physiology: Cellular Circuits

Intercellular coupling provided by gap junctions can elicit and isolate cell circuits by limiting the passage of signal molecules such as Ca^{2+} and IP_3 (Spray & Bennett, 1985; Kirkpatrick & Peifer, 1995; Newman & Zahs, 1997). Intercellular coupling has been implicated in the maintenance of cellular growth and differentiation, synapse formation and the establishment of neuronal circuits (Belliveau et al., 1991; Bennett et al., 1991, Lang et al., 1991; Fulton, 1995; Kandler & Katz, 1995). In neurons, gap junctions are thought to mediate synchrony among active cells. Clusters of neurons are known to be synchronously active, potentially via gap junctions, which may contribute to the formation of Hebbian synapses (Jefferys, 1995). Hebbian synapses are based on the coincident activation of pre- and post-synaptic neurons providing a higher level of plasticity to synaptic inputs through changes in postsynaptic Ca^{2+} (Pennartz, 1997; Skrebitsky & Chepkova, 1998). Gap junctions also appear to have a central role in spreading depression which is a slow depolarization of cerebral neuronal activity which causes prolonged hypoexcitability possibly triggered by Ca^{2+} waves (Nedergaard et al., 1995; Paul, 1995). Gap junction blockers inhibit spreading depression possibly by preventing the passage of IP_3 between cells which stalls the initiation of the Ca^{2+} wave (Nedergaard et al., 1995). These changes in communication between cells can be easily observed through dye-coupling. Recent dye-coupling experiments have implicated gap junctions in the formation of neural circuits, extraneuronal

communication and even as neuromodulators (MacVicar & Dudek, 1980; MacVicar & Dudek, 1981; Gutnick & Prince, 1981; Andrew et al., 1982; Massa & Mugnaini, 1982; Dudek, 1988; Peinado et al., 1993; Yuste et al., 1992, 1995).

2.12 Gap Junction Physiology: Charcot-Marie Tooth Disease

The X chromosome linked form of Charcot-Marie-Tooth (CMT) disease results from a naturally occurring loss of function or premature termination mutation in the gene for Cx 32 (Pericak-Vance et al., 1995; Ionasescu et al., 1996a, 1996b; Ainsworth et al., 1998). CMT is a distal peripheral neuropathy of Schwann cells that involves the degeneration of myelin (Scherer et al., 1995; Spray & Dermietzel, 1995). This disease develops in early childhood and is expressed as light paralysis in the hands and feet which leads to motor function and balance difficulties (Ionasescu et al., 1996a, 1996b). Cx 32 is normally expressed in uncompact myelin with a particular concentration near the nodes of Ranvier and the Schmidt-Lantermann incisures (Balice-Gordon et al., 1998). Recent evidence suggests that Cx 32 may also serve a regenerative role in the peripheral nervous system (Dezawa et al., 1998). Following nerve transection it appears that Schwann cells communicate directly with axons via Cx 32 positive gap junctions. In the case of CMT it seems the passage of vitamins, nutrients and signalling molecules is insufficient and the cells degenerate.

Humans do not require "normal" Cx 32 expression for development since CMT patients have no other clinical symptoms from the lack of functioning Cx 32. Direct sequence analysis of CMT patients has shown numerous mutations in the Cx 32 coding region but these patients are not considered Cx 32 knockouts because the gene is still expressed (Bennett et al., 1994; Paul,

1995; Martini et al., 1998). A recent study found that mutated Cx 32 protein trafficking was severely altered and could accumulate to toxic levels in the cytoplasm of myelinating cells (Deschenes et al., 1997). Cx 32 knockout mice exhibit the demyelination of CMT but the exact disease phenotype is not matched since CMT arises from a mutated not absent form of Cx 32 (Willecke & Haubrich, 1996; Nicholson & Bruzzone, 1997; Temme et al., 1997).

2.13 Gap Junction Physiology: Dye-Coupling

Dye-coupling is an often used technique based on the hypothesis that the incidence of coupling represents the functional state of gap junctions. Low molecular weight dyes such as propidium iodide, biocytin or Lucifer Yellow are injected into cells which are observed for dye flow into neighbouring cells which are presumed to be coupled by gap junctions (Kaneko & Stuart, 1984; Hidaka et al., 1993; Bloomfield et al., 1995; McMahon & Mattson, 1996).

Connexins are selectively permeable to certain dyes but all known connexins will allow passage of biocytin and Lucifer Yellow (Veenstra, 1996).

2.14 Dye-Coupling in the Retina

Dye-coupling is often used to study gap junctions in the retina (Robinson et al., 1993; Penn et al., 1994; Vaney, 1994; Mills & Massey, 1996; Smith & Vardi, 1995; Wingate, 1996). The retina is an outpouching of the embryonic forebrain that is well defined both morphologically and biochemically providing a very simplified model of the brain (Wassle & Boycott, 1991; Hitchcock & Raymond, 1992; Kolb, 1994). Within the retina, gap junctions have been demonstrated between photoreceptors and the inner plexiform layer, between horizontal cells, between amacrine cells and cone bipolars, and between bipolar and ganglion cells (Hampson et

al., 1992; Hidaka et al., 1993; Robinson et al., 1993; Penn et al., 1994; Vaney, 1994; Smith & Vardi, 1995; Mills & Massey, 1996; McMahon & Mattson, 1996; Wingate, 1996; Newman & Zahs, 1997).

2.15 Dopaminergic Modulation of Gap Junctions in the Retina

DA is known to modulate both homologous and heterologous coupling between neurons in the retina (Hampson et al., 1992; Baldrige et al., 1998). Extracellular DA levels vary in response to the level of light intensity (Djamgoz & Wagner, 1992; Baldrige et al., 1998; Xin & Bloomfield, 1999). Horizontal cells produce a light induced response called an s-potential and the spread of these s-potentials is gap junction mediated and is decreased by DA (Lasater & Dowling, 1985; Djamgoz & Wagner, 1992; Vaney, 1994). DA uncouples horizontal cells and rod amacrine cells through a cAMP dependent protein kinase which causes the phosphorylation of connexins and a decrease in open probability of the channel (Lasater, 1987; DeVries & Schwartz, 1989; McMahon et al., 1989; Hampson et al., 1994; Mills & Massey, 1995; Baldrige et al., 1998). In dark conditions, horizontal cell coupling is weak therefore GABA is released which inhibits the release of DA, allowing information to pass to ganglion cells (Dowling, 1986; Xin & Bloomfield, 1999). There is decreased dye-coupling between horizontal cells following both exogenously applied and endogenously released DA (Hampson et al., 1992; Hampson et al., 1994).

2.16 Dopaminergic Modulation of Dye-coupling in the Brain

Dye-coupling has demonstrated dopaminergic modulation of gap junctions in the NA, ST, HIP and neocortex (Baimbridge et al., 1991; Cepeda et al., 1989, 1993; O'Donnell & Grace, 1993; Onn & Grace, 1994; Jefferys, 1995; Rorig et al., 1995; Onn & Grace, 1996). The effect of

DA agonists and antagonists on dye-coupling is not consistent across all regions but there is no doubt that DA affects neuronal coupling in the brain.

Increases in striatal dye-coupling are observed following exposure to apomorphine (D_1/D_2 agonist) and quinpirole (D_2 agonist). Increased striatal coupling is likely mediated by the D_2 receptor since the D_1 agonist SKF 38393 and the D_1 antagonist SCH 23390 did not affect dye-coupling (Onn & Grace, 1994). D_2 drugs such as clozapine (atypical antipsychotic) and haloperidol (typical antipsychotic) will affect the level of dye-coupling in the striatal complex (O'Donnell & Grace, 1995; Onn & Grace, 1995). Specifically, following withdrawal from clozapine and haloperidol administration there was increased coupling in the ACC and following haloperidol there was increased coupling in the ST. Dye-coupling was increased in these regions by approximately 66-71% (Onn & Grace, 1995). 6-OHDA lesions of the substantia nigra, known to cause significant decreases in DA, also increase the incidence of dye coupling in the ST (Cepeda et al., 1989).

In the HIP, administration of the DA agonists apomorphine (D_1/D_2 agonist) or SKF 38393 (D_1 agonist) reduced dye-coupling in the CA1 between pyramidal cells and the D_1 antagonist SCH 23390 reversed this effect (Velazquez et al., 1997). Pyramidal cells in slices of rat cortex and neocortex injected with neurobiotin revealed approximately thirty coupled cells (Rorig et al., 1995; Rorig & Sutor, 1996). Following incubation with DA there was a significantly reduced level of cellular coupling. Further examination revealed that the DA agonists SKF 38393 (D_1 agonist) and quinpirole (D_2 agonist) also led to uncoupling. No attempt was made to determine the

connexin makeup of these gap junctions. These data provide strong evidence for DA receptor mediation over gap junction electrical coupling.

Long term cocaine self-administration is known to produce changes in the level of DA and its transporter. These changes occur in the ACC and ST which have been extensively examined at the behavioural, neurochemical and pharmacological level. DA also has established effects on electrical coupling between neurons in both the ACC and ST. DA mediates nucleus accumbens and striatal dye-coupling and the extent of dye-coupling is used as an indicator of the functional state of gap junctions. Given this strong relationship between DA and gap junctions, it is likely that exposure to cocaine would have effects on electrical coupling. Therefore gap junctions are a possible target site to look for a chronic adaptation that would underlie the reinforcing and addictive effects of cocaine.

Why Examine Cx 32?

Cx 32 is one of the best characterized connexins of the entire connexin family. When gap junction research started, Cx 32 was thought to be the only gap junction protein rather than one of a group of connexin proteins (Hertzberg & Gilula, 1979). Improved study of Cx 32 was not possible until a technique was developed that was detergent and collagenase free (Gros et al., 1983; Hertzberg, 1984). Previous gap junction isolation procedures had too much contamination so an accurate measure of connexin molecular weight was impossible (Hertzberg & Gilula, 1979). These early studies on liver gap junctions revealed a 27,000 kD band with a 47,000 kD aggregate with no glycosylation site (Hertzberg & Gilula, 1979; Hertzberg, 1984). Paul (1986) was the first

to outline the entire amino acid sequence and develop an affinity-purified antibody directed at Cx 32. The predicted molecular weight from the cDNA was 32,007 kD which is how Cx 32 was named though the actual weight is closer to 27,000 kD (Dermietzel et al., 1990). Further study revealed that the Cx 32 antibody designated M12.13 was directed at the 98-124 amino acid sequence of the cytoplasmic loop (Goodenough et al., 1988; Scherer et al., 1995). M12.13 has become the definitive Cx 32 antibody cited in the majority of research involving Cx 32 (Swenson et al., 1989; Naus et al., 1990; Scherer et al., 1995; Stauffer, 1995; Rhee et al., 1996). Overall, Cx 32 is not only the best characterized of the connexins but M12.13 has proven to be extremely reliable across a wide range of species, cell types and techniques.

How to Examine the Cx 32 - Cocaine Relationship?

There are techniques that are best suited to examine changes in Cx 32 following different periods of exposure to cocaine. Immunohistochemistry, Western blot and Northern blot analysis have been extensively used to examine changes in connexin protein and gene expression (Kojima et al., 1995, 1996; Nadarajah et al., 1996; Nagy et al., 1997; Satake et al., 1997; Yoshimura et al., 1998).

Immunohistochemistry is based on the ability of antibodies to bind with high affinity to specific antigens. It can be used to localize antigens to individual cells and even subcellular compartments. The critical factor in immunohistochemistry is fixation which determines the morphology of the tissue and the signal-noise ratio. Several fixatives have been used to try and optimize connexin staining (ex: glutaraldehyde fixation) but there was no discernable effect on

gap junction morphology therefore paraformaldehyde is the standard (Wolburg & Rohlmann, 1995). The immunoperoxidase technique is a three step process that amplifies the antibody-antigen reaction. The primary antibody is bound to its epitope and is coupled to the peroxidase complex by a bridging antibody called a secondary. This non-covalent link between the secondary and tertiary antibody provides the peroxidase with maximum reactivity to the diaminobenzidine (DAB) substrate. The reaction of the peroxidase with DAB causes a colouration at the epitopic site that is viewed by bright-field microscopy. It is the most efficient method for cell specific connexin detection that demonstrates a definite punctate staining (Dermietzel et al., 1990; Kojima et al., 1995; Nadarajah et al., 1996; Nagy et al., 1997). Sections of liver or brain that are pre-absorbed with preimmune serum show no reactivity to M12.13 therefore the antibody is reliable for immunohistochemistry (Goodenough et al., 1988; Scherer et al., 1995).

Western blot analysis involves the discontinuous SDS (sodium dodecylsulfate) polyacrylamide gel electrophoresis (PAGE) of proteins. Proteins are reacted with SDS, which is an anionic detergent, to form negatively charged protein-SDS complexes. When the proteins bind with the SDS they are denatured and solubilized, and form a rod that is proportional in length to their molecular weight (Garfin, 1990). When an electric field is applied these negatively charged complexes can now be separated by molecular weight as they pass through the matrix of the polyacrylamide gel towards the positive electrode (Bier et al., 1983). The polyacrylamide gel is cast as a separating gel topped by a stacking gel each with their own pH. Protein samples are loaded into the stacking gel following boiling and the addition of B-mercaptoethanol which

denatures the proteins. The difference in gel pH causes a localized gradient squeezing the SDS-protein complexes into a thin stack when they pass from the stacking gel to the separating gel (Garfin, 1990). The separating gel acts like a sieve therefore the lower molecular weight proteins pass through the gel faster than larger proteins. After the gel run is complete the proteins trapped in the separating gel are transferred to a nitrocellulose membrane using a semi-dry transfer unit. This membrane traps the transferred proteins (antigens) which can then be probed with an antibody in order to identify the presence of various proteins such as connexin 32.

Western blot analysis is effective when examining connexins because gap junctions are more resistant than nonjunctional membrane to solubilization by detergents (Hertzberg, 1980). Immunoblotting of Cx 32 has been examined in detail (Satake et al., 1997; Yoshimura et al., 1998). As mentioned previously the Cx 32 antibody (M12.13) recognizes an epitope on the cytoplasmic side and is monoclonal however, the autoradiograph of a Cx 32 Western blot will reveal more than a single band at 27,000. The extra bands are due to proteolysis, both endogenous and exogenous and aggregation of Cx 32 during processing for SDS-PAGE (Fallon & Goodenough, 1981). There is a 40-50 kD dimeric form (aggregate polypeptides) of Cx 32 that appears from the application of heat during the SDS PAGE procedure (Henderson et al., 1979; Hertzberg, 1984; Paul, 1986; Goodenough et al., 1988; Dermietzel et al., 1990). The lower weight bands between 10-13kDa are proteolysis products (Henderson et al., 1979; Paul, 1986; Goodenough et al., 1988). Connexins have no extracellular N-linked glycosylation site therefore no glycosylation has been detected (Hertzberg & Gilula, 1979; Paul, 1986; Bennett et al., 1991).

Northern blot analysis is an electrophoretic technique that provides mRNA sizes and an approximate concentration. Total cellular RNA is separated by size using agarose gel electrophoresis in the presence of formaldehyde (the denaturing agent). The gel is then blotted onto filter or nitrocellulose membrane and hybridized for a prolonged period of time with a labelled probe under conditions favouring hybridization then the mRNA can be detected by autoradiography. This technique is not considered sensitive enough for small samples detection of rare mRNA's but is suited for the detection of connexins (Kojima et al., 1995; Yoshimura et al., 1998).

Proposal

This series of studies proposed to look at the potential role of connexin 32 in the reinforcing effects of cocaine self-administration. Various time courses were used to determine if any changes in CX 32 expression were correlated with the physiological conditions seen following cocaine self-administration.

EXPERIMENT 1: *Connexin 32 protein expression in the brain of drug naive rats*

This initial experiment determined which regions of the drug naive rat brain had a detectable level of Cx 32 protein expression. This was a necessary step in order to determine which areas of the brain were best suited to examination following exposure to cocaine self-administration. This experiment also established the basal differences in Cx 32 expression that exist between brain regions.

MATERIALS & METHODS**Subjects**

Male Wistar rats (N=4) from Charles River (Ste-Foy, QC) weighing 300-400g were used. Following a quarantine period of one week, rats were housed by two or three in the vivarium with a 12 hour light dark cycle. Once entered in the experiment, rats were individually housed under a reversed light/dark schedule (lights off: 03:00-15:00). Food and water were available *ad libitum*.

Procedure

All experimental procedures described in this thesis were approved by the Carleton University Animal Care Committee and met the guidelines set out by the Canadian Council on Animal Care (CCAC).

In summary, rats were decapitated and the brains were processed according to the trizol procedure. The following brain regions were dissected: hippocampus, nucleus accumbens,

striatum, thalamus, medulla and cerebellum. Western blot analysis was performed to determine the basal level of Cx 32 protein expression in drug naive rats.

Brain Processing for Western Blot Analysis

Rats were injected with an overdose of somnotol and decapitated. Brains were rapidly removed and dissected on ice. Specified brain regions were flash frozen in dry-ice chilled isopentane and kept in the -80°C freezer until processed. Prior to starting any molecular technique the initial step was the isolation of RNA and protein using Trizol (Mol. Res., OH). Trizol is a phenol-guanidine thiocyanate solution which separates RNA and protein from a brain region homogenate through the addition of chloroform. Brain regions were removed from the freezer and immediately homogenized with a tissue-tearer in the presence of Trizol. Following centrifugation, the RNA was contained in the aqueous phase and the protein was in the organic phase. The remaining steps were followed according to the manufacturer's protocol. At the end of this procedure each dissected brain region had an isolated RNA and protein pellet. The RNA pellet was stored in 75% ethanol at -20°C until further processing. The protein pellet was washed repeatedly in guanidine hydrochloride to remove any traces of phenol and stored in 100% ethanol until further processing. The protein pellet must be completely free of all traces of phenol and chloroform from the Trizol procedure prior to quantification.

Protein Quantification Protocol

Protein pellets were air dried and dissolved in 1% SDS at 60°C for 8 hours. All protein pellets obtained through the Trizol procedure were colourimetrically quantified according to the Lowry method. This method quantifies proteins based on the reaction of Folin-Ciocalteu phenol reagent (BioRad). The first reaction is between the unquantified protein and copper in an alkaline solution and then a subsequent reaction between the copper-treated protein and the Folin reagent. The colour reaction develops from the reaction of the tyrosine and tryptophan amino acids in the unknown proteins. The colour value obtained is compared to the colour value of a known protein (ex: bovine serum albumin) recommended by the manufacturer and derived from a standard curve. Values for the colour are provided by a spectrophotometer (Ultraspec 1000, Pharmacia Biotech) with a maximum absorbance at 750nm.

Western Blot Analysis Protocol

Protein aliquots and a standard (broad-range, BioRad) were diluted 1:1 with 2X/SDS sample buffer and β -mercaptoethanol to 2.5% final concentration. Aliquots were boiled for 5 minutes and immediately loaded into the stacking gel. Aliquots (40 μ g) were separated by SDS-PAGE under reducing conditions on 12.5% polyacrylamide gels containing 0.1% SDS. Gels, nitrocellulose membranes and filter papers were then soaked in Bjerrum Schafer-Nielson buffer (48mM tris, 39mM glycine, 20% methanol, .0375% SDS) for 30 minutes. Proteins in the gels were then transferred to nitrocellulose membrane using a semi-dry transfer unit (BioRad) at 24 volts for 30 minutes. Membranes were soaked in Ponceau S solution (Sigma) for 5 minutes and

gels were stained with Coomassie Blue to confirm a complete protein transfer (see Appendix 1 for details). Membranes were then washed in 10mM PBS and blocked for 1 hour. Antibodies were blocked and diluted in 10 mM PBS pH 7.5 containing 1% heat-denatured casein (Sigma).

Western analysis was performed using monoclonal β -anti-tubulin (1:400, Sigma) overnight on a shaker-table at 4°C. This antibody was probed first as a loading control for the Western blot analysis (see Appendix 1 for details). Membranes were washed in 10 mM PBS for 20 minutes, blocked for 20 minutes and probed with biotinylated goat anti-mouse IgG (1:20,000; Sigma) for two hours on a room temperature shaker-table. Membranes were then washed and blocked as above and probed with ExtrAvidin peroxidase (1:1000; Sigma) for two hours with shaking at room temperature. Immunoreactive bands were visualized on autoradiographic film by chemiluminescence according to the manufacturer's protocol (Boehringer Mannheim). Following completion of the autoradiographs the membranes were washed, blocked, and reprobed with M12.13 overnight following the same protocol as β -anti-tubulin. Prior to starting the experiments it was necessary to determine the signal to noise ratio of M12.13 for Western blot analysis. Figure 2 is an example of a membrane processed through the entire Western protocol with and without M12.13. There was no excess binding visible without the addition of primary other than the expected biotin bands from the secondary and tertiary antibody.

Results

All six of the regions examined displayed some level of Cx 32 protein expression (see Table 2). Regional variations in Cx 32 protein expression were calculated as a rough mean

Figure 2. (A) the broad range standard for the Western Blot which provides the markers for the molecular weights as the proteins separate in the resolving gel (B) Western Blot analysis done with Cx 32 primary on a cocaine time course (C) Western blot analysis done without Cx 32 primary antibody at 20, 10, 5 and 2 μ g.

A

— Myosin
 — β -galactosidase
 — Phosphorylase B
 — Bovine serum albumin
 — Ovalbumin
 — Carbonic anhydrase
 — Soybean trypsin inhibitor
 — Lysozyme
 — Aprotinin

B

C

Region	Connexin 32
hippocampus	**
nucleus accumbens	***
striatum	****
thalamus	****
medulla	*
cerebellum	*

Table 2. Qualitative assessment of basal levels of Cx 32 protein expression in control rats. The * symbol denotes connexin 32 band intensity on the autoradiograph following Western blot analysis.

estimate of N=4 animals. The range of Cx 32 expression was highest in the striatum and thalamus and lowest in the medulla and cerebellum which had little to no expression of Cx 32.

Discussion

These results determined which brain regions had levels of Cx 32 protein expression that could be visualized by Western blot analysis. Previous studies, using immunohistochemistry and *in situ* hybridization, had indicated that Cx 32 would be present in these brain regions (Yamamoto et al., 1989; Naus et al., 1990; Micevych & Abelson, 1991). This study ensured that the level of Cx32 was also detectable by Western blot analysis in order to continue with the cocaine self-administration studies. It is important to note that the presence of Cx 32 protein in these brain regions is not indicative of functional gap junctions.

EXPERIMENT 2: *Connexin 32 protein expression following chronic cocaine****self-administration***

There is an established relationship between DA and gap junction coupling. Given that the inhibition of dopamine reuptake is cocaine's principle mechanism of action it is likely that exposure to cocaine would have effects on connexin protein. Cocaine has differing and regionally specific effects depending on the brain region therefore the four brain regions expressing the highest level of Cx 32 protein expression were examined following long term cocaine self-administration.

MATERIALS & METHODS**Subjects**

Male Wistar rats (N=24) from Charles River weighing 300-400g were used. Care of animals was identical to the procedure summarized in experiment 1.

Procedure

In summary, rats self-administered cocaine for fourteen consecutive days followed by a predetermined time period without access to cocaine. These animals were randomly divided into a 48 hour, a 7 day, and a 21 day group with no access to cocaine. Animals were sacrificed in the late morning of their designated time period. N=12 rats were decapitated and processed according to the trizol protocol. N=6 were perfused and prepared for immunohistochemical analysis. This

study also included additional control rats; N=2 by decapitation and N=4 by perfusion. The following brain regions were examined; hippocampus, nucleus accumbens, striatum, and thalamus.

Food Training

At the start of the experiment rats were food deprived for 24 hours and trained to lever press for a food reinforcer which was a 45 mg Noyes sweetened pellet. The schedule was an FR 1 and rats were required to complete two consecutive successful training periods. A successful session was defined as a minimum of 500 lever presses for food. Water was available throughout the training session(s) and food was ad libitum following completion of the training. Training was carried out in testing chambers similar to those described below.

Surgery

Rats were anaesthetized with Somnotol (60 mg/kg IP) and implanted with a chronic indwelling silastic cannula. Incisions were made on the back at the mid-scapular area and over the left jugular vein on the ventral side of the rat. A pocket was formed and the silastic tubing was slid subcutaneously from the dorsal incision to the ventral opening. The jugular vein was exposed and punctured with a vein pick to allow feeding of the silastic tubing 1mm away from the heart. The vein was then sutured off to stabilize the tubing, the ventral incision was sutured and some antibiotic (Neosporin, Wellcome Med.) was applied. The cannula was secured at the dorsal incision by two sutures anchoring the marlex mesh to the back muscle and the dorsal incision was sutured and a suppository analgesic (Abenol, SmithKline Beecham) was given. The tubing exits

from this dorsal incision and was protected by a spring attached to a swivel. Rats were watched and kept under warm light until they woke up from the anaesthetic.

Testing Chambers

Rats were singly housed and tested within the same chamber. These testing boxes were made of transparent plexiglass (25cm X 25cm X 25 cm) with a steel grid floor. Each chamber contained a counter-balance mechanism for the swivel/tubing, a water bottle, a stimulus light and a retractable lever. The rats were placed in these boxes following surgery and the fluid swivels were attached to the counterbalances. The swivel was then attached to a 10 ml syringe of cocaine by a length of tubing. This syringe was placed in an infusion pump which was activated by a lever press. When the lever was pressed the stimulus light turned on for 20 seconds and signalled that a lever press during this period would not result in an injection of cocaine.

FR 1 Cocaine Self-administration

All self-administration sessions began with a single priming injection and then rats had a maximum of six hours of FR 1 to reach 40 injections (a minimum of 35 injections had to be reached for successful completion of a session). Each lever press resulted in a single injection of cocaine (1.5 mg/kg/0.1 ml injection) over a five second period. Animals were allowed a maximum of forty injections per day over the designated experimental time period. Time off cocaine was done by removing the cocaine syringe from the individual boxes.

Western Blot Analysis

The identical procedure of brain processing for Westerns, protein quantification, and the Western blot protocol were followed (N=14) according to the details outlined in experiment 1.

Densitometry of Autoradiographs

The density of autoradiographs was calculated (Imaging Research Inc.) using three variables: $\text{density} = \log_{10} \text{incident light} / \text{transmitted light}$.

Density is a proportion of incident to transmitted light. Incident light is the actual light which falls on a specimen. Transmitted light is the light which passes through the specimen. Transmittance decreases as the specimen absorbs more of the incident illumination. Therefore as the specimen darkens the density increases. Density was recorded on the sensing element of a non-image forming photomultiplier that responded linearly to a range of illumination intensities. A density reference standard, which maintains constant properties of absorption and reflectance across a range of illumination intensities, was established following the manufacturer's protocol. The density, calculated as illumination induced voltage, is displayed numerically in accord with the digitized sensors which use 256 discrete shades of gray. The gray level is equivalent to the relative optical density. The relative optical density calculated using the Imaging Research system had the additional advantage of being in accord with the perceptual judgement of how dark the specimen was.

Densitometry Presentation

Western blot analysis results are presented as bar graphs with the time course on the abscissa and the relative optical density on the ordinate. Each brain region has its own bar graph. These graphs represent relative optical densities collapsed across an N=3. The autoradiographs are controlled with regards to the darkroom solutions, the length of autoradiograph exposure to the membrane and all were processed on the same day using the same reference density standard. This is a more efficient means of presenting Western blot data rather than showing each photo of Cx 32 and tubulin for each region (see Figure 3).

Brain Processing for Immunohistochemistry

For immunohistochemistry, rats (N=10) were perfused transcardially with 50 mls of 100 mM phosphate-buffered saline (PBS: 100 mM sodium phosphate buffer pH 7.5, 154 mM NaCl) followed by 200 mls of 4% paraformaldehyde in 100 mM PBS. Brains were dissected into anterior, midbrain and posterior and post-fixed for 4 hours in 4% paraformaldehyde in 100 mM PBS at 4°C followed by several washes in 10% sucrose. Brains were then processed through the paraffin embedding procedure and sections were cut at 8 µm on a rotary microtome and mounted on sterile gelatin dipped slides.

Immunohistochemistry Protocol

Sections were deparaffinized, rehydrated and equilibrated in 10 mM PBS (10 mM phosphate buffer, pH 7.5, 154 mM NaCl). Antibodies were diluted in a buffer consisting of 10

Figure 3. A complete set of autoradiographs for N=1 in the hippocampus from an animal that self-administered cocaine for 14 days. Reading from left to right: lane 1 is from a drug naive animal, lane 2 is 48 hours, lane 3 is 7 days, and lane 4 is 21 days without exposure to cocaine. (A) the membrane probed with tubulin (B) the membrane probed with M12.13 for connexin 32.

A

B

mM PBS, 0.3% Triton-X, 3% BSA and 2% lamda carrageenan, pH 7.5. Sections were probed with Cx 32 primary antibody M12.13 (1:10) at 4°C overnight. The following day sections were washed in 10 mM PBS and then labelled with goat anti-mouse IgG (1:100, Sigma) for 2 hrs at room temperature. Sections were then washed and incubated with extravidin peroxidase (1:100, Sigma) for 2 hrs at room temperature. Slides were washed in 10 mM PBS then presoaked for 5 minutes in a solution containing 1 mg/ml diaminobenzidine in 50 mM Tris-HCL, pH 8.0. Slides were then reacted with 0.003% hydrogen peroxide.

Prior to starting the immunohistochemistry it was necessary to determine the signal to noise ratio of M12.13. Figure 4 is an illustration depicting the exact regions where all the photographs were shot across all experiments. Figure 5 is a section of the hippocampus (CA2) processed according to the immunohistochemistry protocol with and without M12.13. There was no visible non-specific staining. These controls were performed for every experiment in all brain regions examined. In addition, to determine if the Cx 32 staining was neuronal or only localized to oligodendrocytes sections were double-labelled with M12.13 and NeuN (a marker for neuronal nuclei). Figure 6 shows an example of double-labelling from each region (see Appendix 2 for details). M12.13 is stained yellow-gold from diaminobenzidine and NeuN is stained blue from BCIP/NBT. Once Cx 32 was localized to both neurons and oligodendrocytes only M12.13 was probed on the sections using a nickel-intensified diaminobenzidine resulting in a purple colour.

Figure 4. Representations from Paxinos & Watson (1986) of the exact locations of the immunohistochemistry photographs. (A) plate 12 illustrates the nucleus accumbens and striatum (B) plate 29 illustrates the CA1, CA2 and CA3 regions of the hippocampus and the dorsomedial thalamus.

A

B

Figure 5. Immunohistochemistry in the CA2 region of the hippocampus (A) without primary antibody, magnification 50x (B) an adjacent section probed with M12.13 for connexin 32, magnification 50x.

A

B

Figure 6. Double labelling of both M12.13 for connexin 32 (yellow-gold) and NeuN for neuronal nuclei (Blue). (A) there are clearly visible connexin 32 positive oligodendrocytes stained yellow-gold and connexin 32 positive neurons which have blue nuclei and yellow cytoplasm in the cells of the CA2, magnification 200x (B) double labelled cells in the CA1, magnification 50x (C) double labelled cells in the CA2, magnification 50x (D) double labelled cells in the CA3, magnification 50x (E) double labelled cells in the ACC, magnification 50x (F) double labelled cells in the ST, magnification 50x (F) double labelled cells in the TH, magnification 50x.

Results

Long term cocaine administration had varying effects on Cx 32 protein expression in the four regions. The largest change was in the hippocampus. Western blot analysis demonstrated a dramatic decrease in hippocampal Cx 32 protein expression at the 48 hr time point which appeared to rebound by day 7 and continued to increase by day 21 but never reached the original control level (see Figure 7). Immunohistochemical analysis showed Cx 32 immunoreactivity in the control hippocampus localized to neurons of the CA1, CA2 and to a lesser extent the CA3 (see Figures 8, 9 & 10). By the 48 hr time point there is a visible increase in Cx 32 signal in the CA1 field and an apparent absence of Cx 32 in both the CA2 and CA3. By seven days there was a gradual return of Cx 32 immunoreactivity in the CA2 and the CA3. The nucleus accumbens also showed a slight decrease in Cx 32 expression at 48 hrs which appeared to return to control levels by day 7 and 21 (see Figure 11). The immunohistochemistry matches the Western blot data with a visible decrease in Cx 32 immunoreactivity at 48 hrs which rebounds by day 7 (see Figure 12). Western blot analysis of striatal Cx 32 revealed a consistent level of Cx 32 at all the time points examined (see Figure 13). Interestingly, immunohistochemistry revealed increased Cx 32 staining at every time point in comparison to control (see Figure 14). Thalamic expression of Cx 32 appeared unchanged from control at all time points in both Western analysis and immunohistochemistry (see Figures 15 and 16).

Figure 7. Connexin 32 expression in the hippocampus following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours, 7 days and 21 days following the last cocaine self-administration session. There is a decrease in hippocampus Cx 32 expression at the 48 hour time point which remains below baseline levels up to the 21 day time point.

Figure 8. Connexin 32 immunoreactivity in the CA1 of the hippocampus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 32x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 50x. There is an apparent increase in CA1 Cx 32 immunoreactivity at both the 48 hour and 7 day time points.

A

B

C

Figure 9. Connexin 32 immunoreactivity in the CA2 of the hippocampus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 50x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 32x. There is an apparent decrease in CA2 Cx 32 immunoreactivity at the 48 hour time point and a return to baseline by the 7 day time point.

Figure 10. Connexin 32 immunoreactivity in the CA3 of the hippocampus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 50x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 50x. There is an apparent decrease in CA3 Cx 32 immunoreactivity at 48 hours with a return of Cx 32 by the 7 day time point.

Figure 11. Connexin 32 expression in the nucleus accumbens following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours, 7 days and 21 days following the last cocaine self-administration session. There is a decrease in nucleus accumbens Cx 32 expression at the 48 hour time point which returns to baseline levels by the 7 day time point.

Figure 12. Connexin 32 immunoreactivity in the nucleus accumbens following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 32x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 32x. There is an apparent decrease in nucleus accumbens Cx 32 immunoreactivity at the 48 hour time point.

A

B

C

Figure 13. Connexin 32 expression in the striatum following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours, 7 days and 21 days following the last cocaine self-administration session. There is no apparent change in striatum Cx 32 expression at any of the time points examined.

Figure 14. Connexin 32 immunoreactivity in the striatum following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 32x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 50x. There is an apparent increase in striatum Cx 32 immunoreactivity at both the 48 hour and 7 day time points.

Figure 15. Connexin 32 expression in the thalamus following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours, 7 days and 21 days following the last cocaine self-administration session. There is no apparent change in thalamus Cx 32 expression at any of the time points examined.

Figure 16. Connexin 32 immunoreactivity in the thalamus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 50x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 32x. There is no apparent change in thalamus Cx 32 immunoreactivity at either of the time points examined.

A

B

C

Discussion

Cocaine withdrawal produces widespread dopaminergic changes and recurring episodes of drug craving. This study evaluated whether withdrawal from long term cocaine self-administration produced changes in Cx 32 protein expression. Western blot analysis and immunohistochemistry revealed decreases in Cx 32 protein expression in the hippocampus and the nucleus accumbens at the 48 hr time point followed by a gradual return to control levels. Dramatic changes in both the hippocampus and nucleus accumbens at the same time point seem to suggest that the 48 hr time point is a critical turning point in cocaine withdrawal. As discussed previously, the nucleus accumbens has been directly linked to the reinforcing effects of cocaine and both the nucleus accumbens and hippocampus have been implicated in cocaine craving (Nestler, 1994; Breiter et al., 1997; Peoples et al., 1997). The immunohistochemistry provided subtle differences in hippocampal Cx 32 protein expression across the CA1, CA2 and CA3 that were not demonstrated by Western blot analysis because these subregions were combined into a single homogenized hippocampus sample. In summary, the CA1 showed an increase and the CA2 and CA3 a loss of Cx 32 expression at the 48 hr time point. The Cx 32 immunoreactivity appeared to be predominantly neuronal. These regional differences are significant given that pyramidal neurons in the CA1 (but not CA3) are spontaneously active and responsive to dopamine and cocaine can increase CA1 electrical activation (Stein & Belluzzi, 1989; Zhai et al., 1997).

The striatum did not show any marked differences at any of the time points examined through Western blot analysis. However, immunohistochemistry showed a roughly equivalent increase in neuronal Cx 32 staining at every time point in comparison to control. It is likely that

the specific region of the striatum chosen to be photographed was an area of increased Cx 32 protein expression but the Western provides the best indication of the entire dorsal striatum. Unlike the nucleus accumbens and hippocampus, the striatum is not associated with the reinforcing or withdrawal effects of cocaine but rather to behavioural activation (Kennedy & Hanbauer, 1983; Bowman & Kuhn, 1996; Thomas et al., 1996; Pederson et al., 1997). Given the short lasting effects of cocaine the immediate activation would be relatively short lived following the last self-administration session therefore it is not surprising that there is a lack of any Cx 32 change at any time point.

Western blot analysis and immunohistochemistry showed no changes in the level of thalamic Cx 32 expression. This result was expected given the differing effects of cocaine on the various thalamic nuclei which would negate each other (Hadfield & Milio, 1992; Hadfield, 1995; Weissenborn et al., 1998; Young & Deutch, 1998). The Western blot collapses across all the subtle differences and provides the best overall measure of Cx 32 in the thalamus. The thalamus, like the striatum, is implicated in the behavioural activation following cocaine. The striatum and the thalamus showed no change in Cx 32 following long term self-administration which demonstrated that the changes were localized to specific brain regions and were not generalized effects appearing randomly in any region (Biegon et al., 1992; Thomas et al., 1996; Bonate et al., 1997).

Experiment 3: *Determine the levels of connexin 32 expression immediately following a cocaine self-administration session*

Results from experiment 2 showed that Cx 32 expression was altered after 14 days of daily cocaine self-administration. This study examined Cx 32 expression before and at the end of a single self-administration session. These results address whether an acute 4 hour exposure to cocaine would exacerbate or attenuate the change from baseline produced by long term exposure to cocaine.

MATERIALS & METHODS

Subjects

Subjects from the same supplier were used and identical care was taken as described in previous experiments.

Procedure

Rats (N=12) self-administered cocaine for fourteen consecutive days according to the protocols outlined in experiment II. These animals were randomly divided into a pre-session group in which the rats were sacrificed 24 hours after their last exposure to cocaine and a post-session group which were sacrificed immediately after their final infusion of cocaine. Animals were sacrificed in the late morning of their designated time period. N=6 rats were decapitated and processed according to the trizol protocol and N=6 were perfused and prepared for immunohistochemical analysis. All the protocols outlined in Experiments I and II for the

preparation and analysis of tissue for Western blot analysis and immunohistochemistry were followed in this experiment.

Results

Long term cocaine self-administration appeared to have the immediate overall effect of increasing Cx 32 protein expression however, there was no significant difference between the pre-session and post-session groups. It seems that only at the 48 hour time point (discussed in experiment II) are there dramatic decreases. In the hippocampus there were no dramatic changes in Cx 32 expression by Western blot analysis suggesting only a slight decrease in Cx 32 in the post session group (see Figure 17). The immunohistochemistry reflected a somewhat different picture. The CA fields showed an increase CA1, CA2 and CA3 Cx 32 neuronal expression at both the pre and post-session time points (see Figures 18, 19 & 20). The nucleus accumbens showed an equivalent increase in Cx 32 expression at both the pre-session and post-session time points (see Figure 21). In support of the Western data, there was an evident increase in Cx 32 immunoreactivity in both the pre and post-session time points (see Figure 22). Western blot analysis of striatal Cx 32 showed no change in expression immediately before or immediately after the cocaine self-administration session in comparison to control levels (see Figure 23). In contrast, striatal immunoreactivity showed an increased but equivalent expression of Cx 32 staining in both the pre and post-session groups (see Figure 24). Thalamic expression of Cx 32 was increased from control in the pre-session group and slightly lower but still elevated from baseline in the

Figure 21. Connexin 32 expression in the hippocampus following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur at the start of the cocaine self-administration session (pre) and immediately following the cocaine self-administration session (post). There is no apparent change in hippocampus Cx 32 expression at either of the pre or post-session time points.

Figure 18. Connexin 32 immunoreactivity in the CA1 of the hippocampus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed immediately after the self-administration session, magnification 32x (C) section taken from an animal sacrificed prior to the start of the next self-administration session, magnification 32x There is an apparent increase in CA1 Cx 32 immunoreactivity immediately prior to and immediately after a self-administration session.

Figure 19. Connexin 32 immunoreactivity in the CA2 of the hippocampus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed immediately after the self-administration session, magnification 32x (C) section taken from an animal sacrificed prior to the start of the next self-administration session, magnification 32x. There is an apparent increase in CA2 Cx 32 immunoreactivity immediately prior to and immediately after a self-administration session.

A

B

C

Figure 20. Connexin 32 immunoreactivity in the CA3 of the hippocampus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed immediately after the self-administration session, magnification 50x (C) section taken from an animal sacrificed prior to the start of the next self-administration session, magnification 50x. There is an apparent increase in CA3 Cx 32 immunoreactivity immediately prior to and immediately after a self-administration session.

Figure 21. Connexin 32 expression in the nucleus accumbens following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur at the start of the cocaine self-administration session (pre) and immediately following the cocaine self-administration session (post). There is an apparent increase in nucleus accumbens Cx 32 expression at both the pre and post session time points.

Figure 22. Connexin 32 immunoreactivity in the nucleus accumbens following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed immediately after the self-administration session, magnification 32x (C) section taken from an animal sacrificed prior to the start of the next self-administration session, magnification 50x. There is an apparent increase in nucleus accumbens Cx 32 immunoreactivity immediately prior to and after a self-administration session in comparison to baseline levels.

A

B

C

Figure 23. Connexin 32 expression in the striatum following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur at the start of the cocaine self-administration session (pre) and immediately following the cocaine self-administration session (post). There is no apparent change in striatum Cx 32 expression at either of the pre or post-session time points.

Figure 24. Connexin 32 immunoreactivity in the striatum following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed immediately after the self-administration session, magnification 50x (C) section taken from an animal sacrificed prior to the start of the next self-administration session, magnification 50x. There is an apparent increase in striatum Cx 32 immunoreactivity immediately prior to and after a self-administration session in comparison to baseline levels.

post-session group (see Figure 25). Increased thalamic immunoreactivity for Cx 32 was evident in both the pre and post-session groups and the increase appeared to be equivalent (see Figure 26).

Discussion

No dramatic differences in Cx 32 expression were observed between the pre-session (start of a single cocaine self-administration session) and post-session (end of a single cocaine self-administration session) groups. However, Cx 32 expression in both these groups showed dramatic changes in comparison to control levels. The changes from control were found on the day following cocaine exposure at a time point that corresponded to what would have been the start of the next cocaine self-administration session.

The hippocampus provided some interesting results. The western blot showed a slight decrease from baseline in Cx 32 expression in the post-session group whereas the immunohistochemistry indicated an increase in the CA1, CA2 and CA3 in both pre and post-session groups. Western blots are very effective for illustrating broad trends in a protein therefore it is likely that there was a downward trend in hippocampal Cx 32 which reached bottom by the 48 hour time point. It remains unknown if the decrease in Cx 32 expression witnessed in the post-session group was due to the long term cocaine use or to the hour (approximately) off cocaine before the animal was sacrificed. It is possible that cells respond immediately to the lack of cocaine and change the rate of Cx 32 synthesis and/or degradation. These results are not inconsistent with the role of the hippocampus in cocaine craving as discussed in Experiment I (Breiter et al., 1997).

Figure 25. Connexin 32 expression in the thalamus following 14 days of cocaine self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur at the start of the cocaine self-administration session (pre) and immediately following the cocaine self-administration session (post). There is an apparent increase in thalamus Cx 32 expression at both the pre and post session time points.

Figure 26. Connexin 32 immunoreactivity in the thalamus following long term cocaine self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed immediately after the self-administration session, magnification 32x (C) section taken from an animal sacrificed prior to the start of the next self-administration session, magnification 50x. There is an apparent increase in thalamus Cx 32 immunoreactivity immediately prior to and after a self-administration session in comparison to baseline levels.

A

B

C

The nucleus accumbens demonstrated increased Cx 32 expression in both the pre and post-session groups through both Western blot analysis and immunohistochemistry. The nucleus accumbens is the central brain region responsible for the reinforcing properties of cocaine and the site of chronic adaptation to drug reinforcement therefore the potential for increased cell coupling during active cocaine use and the beginning of drug craving is not unexpected (Nestler, 1994; Breiter et al., 1997; Peoples et al., 1997). The next time point, examined in Experiment I was 48 hours and by that time there was a decrease in Cx 32 expression.

Western blot analysis of the striatum revealed no changes at the start or the end of a single session which was consistent with the results from Experiment I which also revealed no changes at the 48 hour, 7 day or 21 day time points. Cx 32 immunoreactivity demonstrated an equivalent increase in Cx 32 staining in both the pre and post-session groups in comparison to control. As in Experiment I, it is likely that the region of the striatum photographed was an intense area of increased Cx 32 protein expression but the Western blot illustrates the trend in expression of the entire dorsal striatum. The striatum is the major site for the dopaminergic changes that underlie the behavioural activation of cocaine (Martin-Fardon et al., 1997; Little et al., 1998). Since cocaine is an extremely fast acting drug with short lasting effects on motor activity it is likely that any behaviourally related change of Cx 32 in the striatum was brief. Also, with chronic self-administration there was a decreased level of behavioral activation from the cocaine and the animal was unable to increase their level of cocaine intake since a maximum of forty infusions was set.

Thalamic expression of Cx 32 protein was equally increased at the start and finish of the single session as indicated by both Western blot analysis and immunohistochemistry. The immunoreactivity highlights a potential role for the mediodorsal nucleus of the thalamus which is implicated in the self-administration of cocaine (Weissenborn et al., 1998). Recent exposure to cocaine undoubtedly had excitatory effects on thalamic cells. Dopamine is a known modulator of thalamic cell excitability therefore the potential for increased coupling is likely (Lavin & Grace, 1998). The increase in Cx 32 expression revealed by Western blot, which encompassed all the nuclei, suggested that the increase occurred in the majority of the thalamus.

Experiment 4: *A further examination of the 48 hr time point - minimum exposure to cocaine required to see changes in Cx 32 expression*

This experiment determined if two weeks of cocaine self-administration were necessary to produce the significant changes in Cx 32 protein expression seen at the 48 hour time point in the hippocampus and nucleus accumbens. On the basis of the data from the long term self-administration study the same regions were selected for further examination.

MATERIALS & METHODS

Subjects

Subjects from the same supplier were used and identical care was taken as described in previous experiments.

Procedure

Rats (N=9) self-administered cocaine for either 3, 7 or 14 days and were sacrificed 48 hrs after their last self-administration session (N=3 per time point) according to the trizol procedure outlined in Experiment I. All the protocols outlined in experiment I and II for Western blot analysis and densitometry were followed.

Results

Western blot analysis of the hippocampus revealed a decrease in Cx 32 at the 48 hour time point only after long term cocaine self-administration (see Figure 27). Neither the 3 or 7 days of

Figure 27. Connexin 32 expression in the hippocampus following 3, 7 and 14 days of cocaine self-administration followed by 48 hours of cocaine deprivation. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. There is a decrease in hippocampus Cx 32 expression only after 14 days of cocaine self-administration.

exposure was enough to replicate the 48 hour decrease seen in Experiment II. The nucleus accumbens showed a decrease in Cx 32 protein expression following both 3 and 7 days of exposure but reached its lowest level after 14 days of cocaine self-administration (see Figure 28). There were no significant changes in striatal levels of Cx 32 protein expression at any of the time points examined (see Figure 29). The thalamus demonstrated a dramatic 48 hour decrease following 7 days of cocaine self-administration with the other time points being similar to control (see Figure 30).

Discussion

Two conclusions can be drawn from this study; 1) long term cocaine self-administration has different effects on Cx 32 protein expression than does shorter intervals therefore exposure is a critical variable and 2) these cocaine exposure effects on Cx 32 vary by region.

The hippocampus results at 14 days were consistent with the results from Experiment II. Shorter term exposure of 3 and 7 days was not sufficient to cause a change in Cx 32 protein expression levels. However, the nucleus accumbens not only demonstrated the expected decrease at 14 days but also at 3 and 7 days. These results suggest that Cx 32 in the nucleus accumbens is more vulnerable to changes following exposure to cocaine. Possibly related to the direct role of the nucleus accumbens in the reinforcing effects of cocaine.

Consistent with the results from Experiment II the striatum showed no changes following chronic self-administration nor at the earlier time points. It seems that striatal Cx 32 is not easily affected by cocaine following short term or long term exposure to cocaine. Interestingly, the

Figure 28. Connexin 32 expression in the nucleus accumbens following 3, 7 and 14 days of cocaine self-administration followed by 48 hours of cocaine deprivation. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Following 3, 7 and 14 days of cocaine self-administration there is a decrease in nucleus accumbens Cx 32 expression.

Figure 29. Connexin 32 expression in the striatum following 3, 7 and 14 days of cocaine self-administration followed by 48 hours of cocaine deprivation. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. There is no change in striatum Cx 32 protein expression at any time point examined.

Figure 30. Connexin 32 expression in the thalamus following 3, 7 and 14 days of cocaine self-administration followed by 48 hours of cocaine deprivation. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. There is a decrease in thalamus Cx 32 protein expression at both the 3 and 7 day time points examined.

thalamus results were consistent at the 14 day time point but there was a dramatic decrease in Cx 32 following 7 days of self-administration. The downward trend in Cx 32 protein expression seemed to begin at the 3 day time point which suggests an earlier effect on thalamic Cx 32 than was previously seen in the other regions. This may be related to the role of the thalamus in mediating the analgesic effect of acute cocaine (Dougherty et al., 1990; Shyu et al., 1992). Short term use of cocaine has known analgesic properties and given that gap junctions have been implicated in pain pathways it may be that the thalamus is a potential location for these effects (Leibold & Schwarz, 1993; Zerari, 1998).

Experiment 5: *Examine the effects of an acute injection of cocaine on the protein expression of Cx 32*

This study determined if long term exposure to cocaine was necessary to produce any changes in Cx 32 protein expression. This study also provided an indication of how quickly Cx32 protein was affected given that acute injections of cocaine were so short lasting any change in Cx 32 had to be almost immediate.

MATERIALS & METHODS

Subjects

Subjects from the same supplier were used and identical care was taken as described in previous experiments.

Procedure

Rats were injected I.P. with 15 mg/kg of cocaine and placed in an activity box for the duration of their time course. Cocaine causes significantly increased and sustained hyperactivity therefore, motor activity measures were taken to confirm that the cocaine was accurately injected. Because acutely administered cocaine is quickly absorbed, animals were sacrificed and processed following the Trizol procedure 2 and 6 hours (N=3 per time point) following a single I.P. injection of cocaine. All the protocols outlined in Experiments I and II for the preparation and analysis of tissue for Western blot analysis were followed in this experiment.

Results

Acute cocaine had little effect on Cx 32 protein expression in any of the regions examined. The hippocampus showed no change at either of the time point examined (see Figure 31). Levels of Cx 32 in the nucleus accumbens also remained stable at 2 and 6 hours (see Figure 32). There appeared to a slight increase in striatal Cx 32 protein expression given that the tubulin loading controls were slightly underloaded (see Figure 33). There were no changes or trends apparent in thalamic Cx 32 protein expression at either time point (see Figure 34).

Discussion

Acute cocaine produces different physiological effects from long term cocaine self-administration (Cole et al., 1992; Ambrosio et al., 1996; Baldwin et al., 1998). Cocaine is rapidly absorbed by the body so any physiological change would be almost immediate (Hemby et al., 1995). These results showed no dramatic changes at any of the time points examined. It may be that the 2 hour time point was already too late to reveal a change in CX 32 protein expression although with the exception of the striatum there were no visible trends. Previous results examining long term cocaine self-administration showed very little or no change in striatal Cx 32. This is the first result which indicates a cocaine effect on striatal Cx 32 protein expression. There is a slight increase at 2 hours which is increased at 6 hours. This effect is best examined in relation to the corresponding tubulin levels. Tubulin represents the loading control for the Western blot. Both the 2 and 6 hour quantities of protein were underloaded and yet the Cx 32 expression was

Figure 31. Connexin 32 expression in the hippocampus following an acute injection of cocaine.

Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2 and 6 hours following a single I.P. injection of cocaine (15 mg/kg). There is no change in hippocampus Cx 32 protein expression at either of the time points examined.

Figure 32. Connexin 32 expression in the nucleus accumbens following an acute injection of cocaine. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2 and 6 hours following a single I.P. injection of cocaine (15 mg/kg). There is no change in nucleus accumbens Cx 32 protein expression at either of the time points examined.

Figure 33. Connexin 32 expression in the striatum following an acute injection of cocaine. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2 and 6 hours following a single I.P. injection of cocaine (15 mg/kg). There is a slight increase in striatum Cx 32 protein expression at either of the time points examined.

Figure 34. Connexin 32 expression in the thalamus following an acute injection of cocaine. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2 and 6 hours following a single I.P. injection of cocaine (15 mg/kg). There is no change in thalamus Cx 32 protein expression at either of the time points examined.

increased. This is a very minor effect but it represents the first change in striatal Cx 32. Overall, it seems that changes in Cx 32 expression are slow to appear. Given the results of the previous experiments it is likely that chronic exposure to cocaine is required to produce a change in Cx 32 expression.

Experiment 6: *Examine the effects of an acute injection of a long acting cocaine analogue (WF23) on the protein expression of Cx 32*

Acute exposure to cocaine produced little change in Cx 32 expression. It is likely that a single injection of cocaine was not sufficient to produce changes because cocaine is rapidly metabolized and changes in Cx 32 appear to require extended exposure to cocaine. Cocaine is a short acting drug whereas WF23 is a very long acting drug. WF23 is a cocaine analogue that is metabolically stable therefore WF23 was a better choice to examine the effects of a single acute exposure. The same time points of 2 and 6 hours were examined with the addition of 12 and 24 hours given that WF23 is a longer acting drug.

MATERIALS & METHODS

Subjects

Subjects from the same supplier were used and identical care was taken as described in previous experiments.

Procedure

Rats were injected I.P. with 1.0 mg/kg of WF23 and placed in an activity box for the duration of their time course. Motor activity measures were taken to confirm the drug was accurately injected as WF23 will cause significantly increased and sustained hyperactivity. Animals were sacrificed and processed following the Trizol procedure at 2 hrs, 6 hrs, 12 hrs and 24 hrs (N=3 per time point) following I.P. injection of WF23. All the protocols outlined in

Experiments I and II for the preparation and analysis of tissue for Western blot analysis were followed in this experiment.

Results

There was an immediate decrease in hippocampus Cx 32 expression by the 2 hour time point which then rapidly increased by 6 hours and continued to peak by the 24 hour time point (see Figure 35). The nucleus accumbens revealed a reverse trend in comparison to the hippocampus. There was a continual decrease in Cx 32 at every time point examined in comparison to baseline levels. By the 24 hour time point Cx 32 expression was still decreasing (see Figure 36). Cx 32 expression in the striatum appeared stable at the 2 hour time point followed by a dramatic decrease in Cx 32 at 6 hours which remained far below control levels by the 24 hour time point (see Figure 37). The thalamus, similar to the nucleus accumbens and striatum, revealed decreases in Cx 32 expression at every time point examined (see Figure 38).

Discussion

WF23 had dramatic and long lasting effects on Cx 32 protein expression in all the regions examined. The previous experiment studying the effects of a single acute injection with cocaine produced no significant changes with the exception of a slight increase in striatal Cx 32. The dramatic effect of a single injection of Cx expression was not unexpected given that WF23 is 230X more potent than cocaine at inhibiting dopamine uptake and is much longer acting than cocaine (Bennett et al., 1995; Hemby et al., 1995).

Figure 35. Connexin 32 expression in the hippocampus following an acute injection of WF23.

Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2, 6, 12 and 24 hours following a single I.P. injection of WF23 (1.0 mg/kg). There is an apparent increase in hippocampus Cx 32 protein expression at the 6, 12 and 24 hour time points examined.

Figure 36. Connexin 32 expression in the nucleus accumbens following an acute injection of WF23. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2, 6, 12 and 24 hours following a single I.P. injection of WF23 (1.0 mg/kg). There is an apparent decrease in nucleus accumbens Cx 32 protein expression at the 2, 6, 12 and 24 hour time points examined.

Figure 37. Connexin 32 expression in the striatum following an acute injection of WF23. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2, 6, 12 and 24 hours following a single I.P. injection of WF23 (1.0 mg/kg). There is an apparent decrease in striatum Cx 32 protein expression at the 6, 12 and 24 hour time points examined.

Figure 38. Connexin 32 expression in the thalamus following an acute injection of WF23. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 2, 6, 12 and 24 hours following a single I.P. injection of WF23 (1.0 mg/kg). There is an apparent decrease in thalamus Cx 32 protein expression at the 2, 6, 12 and 24 hour time points examined.

The hippocampus was the only region which displayed an increase in Cx 32 expression. This may be related to the role of the hippocampus in seizure epileptogenesis (Stein & Belluzzi, 1989; Change et al., 1998). CA1 neurons show increased firing in the presence of both dopamine and cocaine. Therefore it is likely that a drug which is 230X more potent than cocaine at affecting dopamine reuptake would have an immense effect on CA1 neurons. It is also established that synchronous firing in the hippocampus is dopamine modulated and possibly coordinated by gap junctions so any change in dopamine would affect electrical coupling (Perez-Velazquez et al., 1997).

The nucleus accumbens, the site of the reinforcing effects of cocaine, and striatum, the site for cocaine's behavioural activation, are two brain regions which are heavily innervated by dopamine (Nestler et al., 1994; Thomas et al., 1996; Peoples et al., 1997). Both these regions revealed immediate changes in Cx 32 expression far below baseline which continued even 24 hours after a single injection. This suggests that the highly potent cocaine analogue WF23 had an immediate effect on Cx 32 protein expression. In Experiment II and IV there were decreases in nucleus accumbens Cx 32 48 hrs after last exposure to cocaine. These results suggest that decreases in nucleus accumbens Cx 32 were not only related to cocaine withdrawal 48 hours later but could also be seen within hours of a single drug injection. This experiment provided the first dramatic change in striatum Cx 32 expression. Acute cocaine produced a very minor increase in Cx 32 but acute WF23 showed drastically decreased levels in Cx 32 in comparison to baseline. This suggests that the striatum requires a substantial change in dopamine levels before Cx 32 levels are affected.

Cx 32 in the thalamus, unaffected by acute cocaine, produced an immediate decrease in Cx 32 expression which continued to decrease even 24 hours after the injection. In Experiment IV, Cx 32 expression in the thalamus was shown to be more sensitive to short term (3 and 7 day) exposure to cocaine so this was additional evidence for a drastic depletion of Cx 32 expression which occurred within hours. Previously, the acute cocaine results from Experiment V suggested that chronic exposure was required to produce a change in Cx 32 protein expression but this is clearly not true.

Experiment 7: *Determine if changes in CX 32 protein expression are specific to cocaine or common to any drug of abuse*

This study determined if the changes in CX 32 expression observed following long term cocaine self-administration were unique to cocaine or general to any drug of abuse. Heroin was chosen because it has unique physiological and neurotransmitter effects which are distinct from cocaine. This study also demonstrated the effects of long term heroin self-administration on Cx 32 protein expression.

MATERIALS & METHODS

Subjects

Subjects from the same supplier were used and identical care was taken as described in previous experiments.

Procedure

Rats (N=12) self-administered heroin (25 µg/kg) for 14 consecutive days in accordance with the same procedures as the long term cocaine self-administration experiment followed by either 48 hours or 7 days without access to heroin. N=6 animals were sacrificed and processed according to the trizol protocol and N=6 were perfused and prepared for immunohistochemistry (N=3 per time point). The following brain regions were examined; hippocampus, nucleus accumbens, striatum, and thalamus. All the protocols outlined in Experiments I and II for the

preparation and analysis of tissue for Western blot analysis and immunohistochemistry were followed in this experiment.

Results

Western blot analysis of Cx 32 protein expression following long term heroin self-administration indicates that the changes were different from those seen following chronic cocaine. Following long term heroin self-administration there were no dramatic changes in hippocampal Cx 32 protein expression (see Figure 39). Immunohistochemistry revealed increased Cx 32 staining in the CA1 and CA2 with a slight increase in CA3 at the 48 hr time point. By day 7 CA1, CA2 and CA3 were demonstrating a continual increase in Cx 32 immunoreactivity (see Figures 40, 41 & 42). Expression of Cx 32 in the nucleus accumbens showed a slight decrease at the 48 time point which seemed to stabilize at control levels by day 7 (see Figure 43). Immunohistochemistry supports the western data (see Figure 44). Both Western analysis and immunoreactivity demonstrate a decrease in striatal Cx 32 protein expression at both the 48 hr and 7 day time point (see Figures 45 & 46). Western data from the thalamus show increased Cx 32 at 48 hrs with a subsequent decrease at 7 days to near control levels (see Figure 47). Immunohistochemistry demonstrated the same pattern of Cx 32 expression as the Western Blot (see Figure 48).

Figure 39. Connexin 32 expression in the hippocampus following 14 days of heroin self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours and 7 days following the last heroin self-administration session. There is no apparent change in hippocampus Cx 32 protein expression.

Figure 40. Connexin 32 immunoreactivity in the CA1 of the hippocampus following long term heroin self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 50x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 125x. There is an apparent increase in CA1 Cx 32 immunoreactivity at both the 48 hour and 7 day time points.

A

B

C

Figure 41. Connexin 32 immunoreactivity in the CA2 of the hippocampus following long term heroin self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 50x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 125x. There is an apparent increase in CA2 Cx 32 immunoreactivity at both the 48 hour and 7 day time points.

A

Figure 42. Connexin 32 immunoreactivity in the CA3 of the hippocampus following long term heroin self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 125x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 125x. There is a slight increase in CA3 Cx 32 immunoreactivity at both the 48 hour and 7 day time points.

Figure 43. Connexin 32 expression in the nucleus accumbens following 14 days of heroin self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours and 7 days following the last heroin self-administration session. There is an apparent decrease in nucleus accumbens Cx 32 protein expression at the 48 hour time point.

Figure 44. Connexin 32 immunoreactivity in the nucleus accumbens following long term heroin self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 50x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 50x. There is a decrease in nucleus accumbens Cx 32 immunoreactivity at the 48 hour time point which returns to baseline levels by the 7 day time point.

A

B

C

Figure 45. Connexin 32 expression in the striatum following 14 days of heroin self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours and 7 days following the last heroin self-administration session. There is an apparent decrease in striatum Cx 32 protein expression at the 48 hour and 7 day time points.

Figure 46. Connexin 32 immunoreactivity in the striatum following long term heroin self-administration. (A) example of a section from a drug naive animal, magnification 50x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 50x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 50x. There is a decrease in striatum Cx 32 immunoreactivity at both the 48 hour and 7 day time points.

Figure 47. Connexin 32 expression in the thalamus following 14 days of heroin self-administration. Bars represent the relative optical density obtained from (N=3 animals, n=2 replicates) Western blot analysis probing for tubulin and connexin 32. Control animals are drug naive. Time points occur 48 hours and 7 days following the last heroin self-administration session. There is an apparent increase in thalamus Cx 32 protein expression at the 48 hour time point followed by a decrease at the 7 day time point.

Figure 48. Connexin 32 immunoreactivity in the thalamus following long term heroin self-administration. (A) example of a section from a drug naive animal, magnification 125x (B) section taken from an animal sacrificed 48 hrs after the last injection of cocaine, magnification 125x (C) section taken from an animal sacrificed 7 days after the last injection of cocaine, magnification 125x. There is an increase in thalamus Cx 32 immunoreactivity at the 48 hour time point which returns to near baseline levels by the 7 day time point.

Discussion

Long term heroin self-administration proved to have very different effects on Cx 32 than cocaine even though both experiments followed the same time course of withdrawal. Examining the Western blots from Experiment II and this study demonstrate that the changes in the cocaine time course are unique to cocaine and not general to any drug of abuse. Unlike cocaine, there is not a clearly defined connection between heroin and the hippocampus. It has been suggested that animals will directly self-administer heroin into the hippocampus but more evidence is needed (Stevens et al., 1991). The hippocampus revealed no changes following exposure to heroin but the cocaine exposure demonstrated a dramatic decrease in Cx 32 at the 48 hour time point. The nucleus accumbens was the only region in which both heroin and cocaine changes in Cx 32 were similar. Both drugs showed a decrease at the 48 hour time point followed by a gradual return to baseline by 7 days. These results are not surprising given that previous studies have shown that the level of dopamine released in the nucleus accumbens following heroin administration was similar to the level following cocaine (Wise et al., 1995). The striatum, which was consistently unaffected by cocaine, showed a continual decrease in Cx 32 expression at both the 48 hour and 7 day time point following exposure to heroin. These results may reflect the change in dopamine levels through GABAergic inhibition. The striatum is one region which has a high concentration of GABA (Devine & Wise, 1994). The thalamus contains a high concentration of opiate receptors so it is interesting to note that there was an increase in Cx 32 protein expression at the 48 hour time point which appeared to return to baseline by day 7. This is very similar to the changes seen in the hippocampus following long term cocaine self-administration. Overall, long term heroin

self-administration produced changes in Cx 32 protein expression that would require further examination. For this study the results were to demonstrate a unique heroin pattern of Cx 32 changes.

Experiment 8: *Northern blot analysis of Cx 32 mRNA in the hippocampus following long term cocaine self-administration.*

One of the most dramatic results obtained from the cocaine studies was the almost complete disappearance of Cx 32 protein in the hippocampus 48 hours after long term cocaine self-administration. In order to examine Cx 32 mRNA expression it was necessary to design and create a riboprobe. The earlier study examined Cx 32 protein expression and now with the creation of a Cx 32 riboprobe this result can be re-examined by looking at Cx 32 mRNA expression. All the techniques have their various shortcomings but the combination of Western Blot analysis, immunohistochemistry and Northern Blot analysis suggests that there are real changes taking place.

Materials & Methods

Subjects

Subjects from the same supplier were used and identical care was taken as described in previous experiments.

Procedure

In summary, rats were decapitated and the brains were processed according to the trizol procedure. The hippocampus was dissected and flash frozen in accordance with the protocols

described in Experiment I. Northern blot analysis (N=2) was performed to determine the level of Cx 32 mRNA expression in drug naive and long term cocaine self-administration rats.

Brain Processing for Northern Blot Analysis

Rats were injected with an overdose of somnotol and decapitated. Brains were rapidly removed and dissected on ice. Brain regions were flash frozen in dry-ice chilled isopentane and kept in the -80°C freezer until processed. Prior to starting any molecular technique the initial step was the isolation of RNA and protein using Trizol (Mol. Res., OH). Trizol is a phenol-guanidine thiocyanate solution which separates RNA and protein from a brain region homogenate through the addition of chloroform. The hippocampus was removed from the freezer and immediately homogenized with a tissue-tearer in the presence of Trizol. Following centrifugation, the RNA was contained in the aqueous phase. The remaining steps were followed according to the manufacturer's protocol. At the end of this procedure each hippocampus had an isolated RNA pellet. The RNA pellet was stored in 75% ethanol at -20°C until further processing.

Creation of a Non-Isotopic Riboprobe for Cx 32

The pGEM-3Z Cx 32 vector (0.5 µg/µl) was provided by Dr. Paul (Paul, 1986). It was known that the Cx 32 cDNA was in the EcoRI site and the orientation was transcription with T7 produces sense RNA. pGEM-3Z Cx 32 was digested with EcoRI to produce a linearized template. AvrII and NarI were chosen as the restriction enzymes since they do not cut the pGEM-3Z vector but are able to cut the inserted Cx 32 cDNA. AvrII produced the sense strand

and NarI the antisense strand. The riboprobes were transcribed using a biotinylation transcription kit (Boehringer Mannheim) following the protocol provided by the manufacturer. Labelled Cx 32 sense or antisense riboprobes had a final concentration of 20ng/ml and were stored at -70°C until used.

Northern Blot Analysis Protocol

The agarose gel (containing formaldehyde) was poured and then the RNA samples were prepared following the formaldehyde denaturation procedure. The RNA samples were heated at 55°C for 30 minutes, placed on ice for 2 minutes and then left to reach room temperature. Following the values of the RNA quantification obtained from the spectrophotometer reading all wells were prepared to contain 10 µg of RNA. Samples were volume adjusted with loading buffer. The electrophoretic unit ran for approximately three hours at 50 volts. Gels were then stained on a room temperature shaker-table for 30 minutes in 5 µg/ml ethidium bromide and then destained in running water for 60 minutes. The gel was then placed on a UV table exposed to UV light for 45 seconds and a picture was taken. The picture displayed two upper bands which were sRNA and a lower band which was tRNA. Gels were then transferred to membrane overnight using the capillary action transfer of filter paper and paper towels. On the second day, the gel was checked on the UV table to assure that all the RNA had transferred to the membrane. The membrane was then heated to 80°C for 30 minutes and then exposed to UV light, nucleic acid side down, for 2 minutes. Membranes were then prehybridized (buffer from Amersham, IL), hybridized with the probe (4 µl of probe per ml of hybridization solution) and blocked overnight

at 50°C in 10mM PBS with 1% heat-denatured casein. The membranes were then incubated for 1 hour in blocking solution containing anti-fluorescein-AP FAB fragments (1:5000). Following the manufacturer's protocol detection of the Cx 32 probe was then completed using a chemiluminescent substrate for alkaline phosphatase called CSPD (Boehringer Mannheim) at 1:100.

Results

Northern blot analysis of the hippocampus revealed a decrease in Cx 32 mRNA expression at the 48 hour time point followed by an increase above baseline levels at the 7 day time point and even higher still at the 21 day time point (see Figure 49).

Discussion

The levels of Cx 32 mRNA expression demonstrated in this study were in agreement with the protein levels seen in Experiment II. In review, following long term cocaine self-administration there was a decrease in Cx 32 protein expression 48 hours after the animal's last exposure to cocaine. By 7 days after the last injection of cocaine, Cx 32 protein levels were starting to return to baseline and were even more increased but still below baseline at the 21 day time point. Cx 32 mRNA expression also revealed a dip at the 48 hour time point but increased beyond baseline at both the 7 and 21 day time points. The 48 hour time point, which revealed decreases in both mRNA and protein expression of Cx 32, is somehow connected with DNA transcription. The techniques necessary to confirm a transcriptional problem were unavailable. At the 7 and 21 day time points there was a gradual return of Cx 32 protein whereas the mRNA

Figure 49. Connexin 32 mRNA expression in the hippocampus following 14 days of cocaine self-administration. Bars represent the ratio of connexin 32 to GAPDH expressed in optical density units obtained from (N=2) Northern blot analysis probing for connexin 32 and GAPDH. Control animals are drug naive. Time points occur 48 hours, 7 days and 21 days following the last cocaine self-administration session. There is a decrease in Cx 32 mRNA expression at the 48 hour time point which increases above baseline levels at both the 7 and 21 day time points.

Cx 32/GAPDH Relative Optical Density

expression came back strongly, far surpassing baseline levels. There was an increase in DNA transcription but the majority of RNA was remaining in RNA form, likely in the trans golgi, and not being translated into protein. Cocaine withdrawal may cause a translational block possibly through the activation of protein synthesis inhibitors. The correlation between these mRNA and protein changes and physiological events, such as craving, require further investigation.

General Discussion

Dopaminergic transmission is critically involved in the reinforcing effects of cocaine. Withdrawal from cocaine causes alterations in synthesis enzyme levels, transporter levels and receptor levels (Hubner & Koob, 1990; Depoortere et al., 1993; Smith et al., 1995; Thomas et al., 1996; Koob & LeMoal, 1997; Graziella De Montis et al., 1998). The consequences of cocaine self-administration on chemical neurotransmission have been examined in great detail but there is no research into the effects of cocaine on electrical coupling. Gap junctions represent a major route of cell to cell communication and are widely dispersed throughout the peripheral and central nervous systems (Paul, 1986; Kandler & Katz, 1995; Bruzzone et al., 1996b; Nadarajah et al., 1996). It is well established that gap junctions between neurons are sensitive to modulation by dopamine therefore cocaine is likely to have an effect on electrical coupling (Cepeda et al., 1989; O'Donnell & Grace, 1995; Onn & Grace, 1995; Perez Velazquez et al., 1997). As discussed in the introduction the basic goal of drug self-administration research is to try and understand what causes the shift between occasional drug use to the loss of behavioural control and drug-seeking that signal addiction. This shift may be accounted for by alterations in gap junction communication. To address this possibility, connexin 32 expression was examined on a withdrawal time course following long term and acute cocaine self-administration. This was the first series of studies to demonstrate that an *in vivo* pharmacological manipulation (cocaine self-administration) can affect the expression of gap junction proteins.

There are widespread changes in the mesocorticolimbic system following withdrawal from long term cocaine self-administration (Koob et al., 1997; Zhang et al., 1998). It is quite

difficult to correlate the changes in Cx 32 with the physiological changes associated with withdrawal. Studies suggest that immediately following long term self-administration and up to four days later there is increased mesolimbic extracellular dopamine, increased dopamine transporters and decreased dopamine receptive neurons in the nucleus accumbens (Weiss et al., 1992; Cerruti et al., 1994; Pilotte et al., 1994; Kuhar & Pilotte, 1996). Dopamine transporters are increased immediately following cessation of cocaine and remain increased for up to three days, after which the level of transporter drops to 33% of the baseline and remains reduced for months. The time points examined in Experiment II and III (pre-session, post-session, 48 hours, 7 days and 21 days after exposure to cocaine) would fall within these boundaries which delineate cocaine withdrawal. The data revealed a fairly uniform increase in Cx 32 expression 24 hours (pre-session) following last exposure to cocaine. By the 48 hour time point there were protracted decreases in Cx 32 protein expression in the nucleus accumbens which remained below baseline levels up to 21 days later. There was no change in the striatum or thalamus. It is quite significant that changes were seen in the nucleus accumbens but not in the striatum given that both regions are responsive to dopaminergic stimulation and would therefore be affected by cocaine. This suggests that the changes in Cx 32 during withdrawal are related more to the reinforcing effects of cocaine associated with the nucleus accumbens rather than the stereotypic behavior associated with the striatum. Like the nucleus accumbens, the hippocampus had decreased Cx 32 protein expression by the 48 hour time point; expression was increased at 7 and 21 days, but still remained below baseline levels. The pattern of Cx 32mRNA mirrored the changes in Cx 32 protein in the hippocampus. If the hippocampus is divided into subregions it seems that there is

increased Cx 32 in the CA1 and decreased Cx 32 in the CA2 and CA3 during cocaine withdrawal. Given that spontaneous firing in the CA1 is dopamine modulated, and that it is CA1 neurons which have been implicated in the generation and spread of seizure activity in the brain, this regional increase of Cx 32 becomes interesting (Stein & Belluzzi, 1989; Perez Velazquez, 1997; Zhai et al., 1997). These results are the first demonstration that cocaine alters gap junction protein expression in the nucleus accumbens and hippocampus. At this point it cannot be determined whether cocaine withdrawal affects gap junction function, but these dramatic changes in Cx protein expression suggest that the assembly of gap junctions would be disrupted.

The central nervous system is differentially affected by acute versus long term cocaine self-administration. There are qualitative and quantitative differences at the level of physiological responses such as heart rate and blood pressure and also at the molecular level in gene and protein expression (Graybiel et al., 1990; Cole et al., 1992; Hope et al., 1994; Ambrosio et al., 1996). A single acute injection of cocaine produced no dramatic change in Cx 32 protein expression in any of the regions examined (Experiment V). This study would appear to indicate that changes in Cx 32 were dependent on chronic exposure to cocaine. However, an examination of the WF23 results clearly shows that changes can appear within 2 hours of a single acute injection (Experiment VI). This suggests that levels of Cx 32 protein can respond rapidly to drug exposure but a single exposure to cocaine may not be potent enough. For example, Western and Northern blot analysis of the hippocampus revealed a drop in Cx 32 protein and mRNA expression at the 48 hour time point. These changes in Cx 32 discussed in Experiments II, III and VIII represent a chronic

change that arises only from repeated exposure to cocaine. Fewer days of self-administration (Experiment IV) had no affect Cx32.

The vulnerability of Cx 32 to changes by cocaine appears to be regionally specific. As discussed above, hippocampal Cx 32 alterations require repeated exposure to cocaine. The nucleus accumbens was unaffected by acute cocaine exposure, but there were decreases seen at the 48 hour time point following 3,7 and 14 days of cocaine self-administration. This may be related to differences in the dopaminergic innervation of the nucleus accumbens and hippocampus. The nucleus accumbens is more highly innervated than the hippocampus and may therefore be affected by cocaine exposure more quickly.

The heroin experiments provided a necessary contrast to the cocaine results. It was important to demonstrate that the changes in Cx 32 protein expression following long term cocaine self-administration were unique to cocaine and not generalized to any drug of abuse. It would seem that the most dramatic changes in Cx 32 are localized to the nucleus accumbens and thalamus, which are associated with the reinforcing aspects of cocaine and heroin, respectively.

Despite all the research conducted in the area of cocaine self-administration there is still no effective treatment for cocaine addiction. Neither is there a clearly defined change in brain neurochemistry which would explain the shift from occasional drug use to addiction. Electrical coupling is an unexplored area in the cocaine literature even though gap junctions are widespread throughout the brain. This series of studies represents a completely new way of examining cocaine self-administration and the changes in the brain that occur following cocaine withdrawal.

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Appendix 1

Control Measures for Western Blot Analysis

Prior to starting each Western blot analysis it was important to determine if the gel run was satisfactory and if the gel transferred properly to the membrane. Ideally the lanes should be loaded with the same quantity of protein and all lanes should have transferred equally to the membrane. One method of examining the gel run was to Commassie Blue stain the gel. This technique showed the protein breakdown by molecular weight and provided a rough indication of whether the lanes were evenly loaded (see Figure 50). Gel A is unevenly loaded indicated by the varying shades of blue on the gel and Gel B is perfectly loaded with all lanes apparently equal in concentration indicated by the even blue staining across all lanes. However, once this stain has been done this gel can no longer be transferred to membrane for Western blot analysis so it becomes necessary to run the same gel again. Another method was to immediately transfer the gel to membrane and then stain the nitrocellulose membrane with Ponceau S. The stained membrane was similar in appearance to the stained gel (only pink in colour) and provided an indication of both the gel run and the uniformity of protein loading in each lane. The gel was then stained with Commassie Blue to assure that most of the protein had transferred to the membrane. In this case the stained gel appeared almost blank with slight staining at the outer edges. If anything looked discrepant the membrane was eliminated and the electrophoresis was repeated. Once the stained membrane appeared equal the membrane was probed with tubulin which was used as a second, more definitive, indicator of the uniformity of protein loading. The autoradiographs revealed a more precise quantification of protein loading and if the tubulin lanes appeared equal the

membrane was re-probed for Cx 32. This meant that any differences seen in Cx 32 would be real changes in Cx 32 protein expression and not due to any loading errors.

Figure 50. Commassie Blue stain of SDS-PAGE gels. Lane numbers run from right to left (A) an unevenly loaded gel - Lane 1: broad range molecular weight standard, Lane 2: 5 μg , Lane 3: 10 μg , Lane 4: 15 μg , Lane 5: 20 μg , and Lane 6: 25 μg of protein loaded (B) an evenly loaded gel - Lane 1: broad range molecular weight standard and Lanes 2-6: each contain 15 μg of protein.

A

B

Appendix 2

Double-Labeling Protocol

Sections were deparaffinized, rehydrated and equilibrated in 10 mM PBS (10 mM phosphate buffer, pH 7.5, 154 mM NaCl) for 15 minutes. Sections were then soaked in 20% glacial acetic acid for 10 minutes to reduce all endogenous phosphatase activity which reduces non-specific staining in the presence of alkaline phosphatase. Sections were then re-equilibrated in 10 mM PBS for 30 minutes. Antibodies were diluted in a buffer consisting of 10 mM PBS, 0.3% Triton-X, 3% BSA and 2% lamda carrageenan, pH 7.5. Half the sections were probed with Cx 32 monoclonal antibody M12.13 (1:10) and the other half were probed with a monoclonal neuronal nuclei marker NeuN (1:100, Chemicon) at 4°C overnight. The following day all sections were washed in 10 mM PBS and then labelled with goat anti-mouse IgG (1:100, Sigma) for 2 hrs at room temperature. Sections which were probed with M12.13 were then washed and incubated with extravidin peroxidase (1:100, Sigma) at 4°C overnight. Sections which were probed with NeuN were then washed and incubated with alkaline phosphatase (1:100, Sigma) at 4°C overnight. The tertiary was left on overnight to ensure complete saturation of the secondary binding sites. The next day all slides were washed in 10 mM PBS and then probed with the second primary antibody. Slides originally probed with M12.13 were then exposed to NeuN and vice-versa. Primary antibodies were left on overnight at 4°C. The following day all sections were washed in 10 mM PBS and then labelled with goat anti-mouse IgG (1:100) for 2 hrs at room temperature. Sections which were re-probed with M12.13 were then washed and incubated with

extravidin peroxidase (1:100) and sections which were re-probed with NeuN were then washed and incubated with alkaline phosphatase (1:100) at room temperature for 2 hours. All slides were then washed in 10 mM PBS for 15 minutes and then sections were covered in BCIP/NBT (BCIP/NBT substrate tablet, Sigma) solution for 6 minutes. This colourized the extravidin alkaline phosphatase blue which labelled NeuN. All sections were then washed in 10 mM PBS and then presoaked for 5 minutes in a solution containing 1 mg/ml diaminobenzidine in 50 mM Tris-HCL, pH 8.0. Slides were then reacted with 0.003% hydrogen peroxide which colourized the extravidin peroxidase brown and labelled Cx 32.