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**The Effect of Heroin Self-Administration and Withdrawal on
Connexin 32 Expression : *in vivo* Pharmacological Manipulation of
Gap Protein Expression**

**By
Tara L. Crowder**

A thesis submitted to the Faculty of Graduate Studies
and Research in Partial fulfilment of the requirements for the degree of
Master of Science

Department of Psychology
Carleton University
Ottawa, Canada
Oct., 1998

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The undersigned hereby recommend to
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acceptance of the thesis submitted by

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Abstract

The neuroadaptive mechanisms that underlie drug addiction are largely unknown. Our lab has recently provided evidence that markers of gap junctional communication may be altered during cocaine self-administration (Bennett, et al. 1998). The current project was designed to determine the effect of another drug of abuse with a different mechanism of action, heroin, on the expression of the gap junction protein Cx32. To this end Cx32 protein expression was examined in both Western blot analysis and immunohistochemistry. Male Wistar rats (n=28) were implanted with chronically indwelling jugular cannulae. The implanted rats were given access to heroin 24 hours a day for a period of 2 weeks. Animals were then sacrificed 15-min., 24-hr, 48-hr, and 1-week following their last injection of heroin. Drug naïve male littermates (n=8) were used as age matched controls. The medulla, hypothalamus, thalamus, nucleus accumbens, and dorsal striatum were dissected from decapitated tissue and were flash frozen in dry-ice chilled isopentane for subsequent protein extraction and Western blot analysis. For immunohistochemistry the tissue was obtained through perfusion with 4% paraformaldehyde and was post-fixed for 4 hours. Western blot analysis indicated regionally specific decreases in Cx32 protein expression. Significant differences in the Cx32:tubulin indices were obtained in the medulla ($p<0.005$) and the hypothalamus ($p<0.005$). Curiously, no changes in Cx32 immunoreactivity were observed. Changes were found in c-Fos immunoreactivity and in Rat IgG. Increases in Rat IgG were observed in the heroin and heroin withdrawal tissue possibly indicating blood brain barrier dysfunction. As a result false-positive staining in Cx32 immunoreactivity could not be ruled out. The observed decrease in Cx32 protein expression may further implicate gap junction mediated intercellular communication in the addictive process.

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The effect of heroin self-administration and withdrawal on connexin 32 expression: *in vivo* pharmacological manipulation of gap junction protein expression.

Overview

The neurobiological mechanisms that underlie drug addiction are largely unknown. Presumably, drugs such as cocaine and heroin produce changes in brain, which lead to emotional and physiological responses that perpetuate drug-taking behaviour. For the most part, research has focused on the effect of drug exposure on adaptations of chemical synaptic transmission (see Nestler, 1997 for review). These studies have demonstrated alterations in receptor proteins, ion channels, and intracellular messengers (Nestler, 1997). However, our lab has recently found that markers of gap junction communication are also changed by extended cocaine exposure. Bennett et al. (1998) demonstrated plastic changes in the expression of the gap junction protein connexin 32 (Cx32) following two weeks of daily cocaine self-administration (submitted). These data have prompted us to hypothesize that gap junction-mediated intercellular communication plays a role in the addictive process.

In the past decade, research has revealed not only the molecular structure but also the dynamic nature of gap junction channels (Peracchia, and Wang, 1997; Bruzzone et al. 1996a; Bruzzone et al. 1996b; Wolburg, and Rohlmann, 1995; and Bennett et al. 1991). Gap junctions have been found to be responsive to changes in neurotransmitter levels, second messengers, hormones, pH, and voltage (Burghardt et al. 1995; Rorig, 1995; Onn, and Grace, 1994; Berthoud et al. 1991; Church, and Bambridge,

1991; and Obaid et al. 1983). Furthermore, several pharmacological agents can induce modifications in gap junction-mediated communication (Ren, and Ruch, 1996; and O'Donnell, and Grace, 1995).

We propose that alterations in gap junction communication may be involved in the neuroadaptive mechanisms underlying both drug dependence and withdrawal. The objective of the current research project was to examine the effect of opiate dependence and withdrawal on the expression of the gap junction protein Cx32.

Introduction

A number of issues will be discussed within this review. First, I will briefly describe the morphology of gap junctions and present evidence for gap junction-mediated communication within the brain. Second, I will discuss the plasticity of this communication. Third, I will consider the effect of cocaine self-administration on Cx32 expression. Ultimately, I will examine the relevance to heroin dependence and withdrawal.

Gap junctions allow for the exchange of ions and small molecules such as nutrients, metabolites, and second messengers (see Bruzzone et al. 1996b for review). The basic structure of these channels was first inferred by Makowski et al. 1977, by analyzing x-ray diffraction patterns recorded from isolated mouse liver gap junctions. It is now known that gap junctions are comprised of two hemichannels or connexons that join in the extracellular matrix to form the complete cell-cell pathways. Each connexon is composed of six integral membrane proteins or connexins arranged in a ring around a

central pore (Yeager, and Nicholson, 1996). Figure 1 illustrates the morphology of a connexon (reproduced from Bruzzone et al. 1996b).

To date, 15 distinct connexin proteins have been isolated and cloned from different tissues and species (see: Spray, 1996, for a review). The proteins share a similar structure and function within the cell. Sequence analysis indicates that the genes encoding the connexin proteins belong to a supergene family (Spray, 1996). The different connexin species are identified with the designation Cx{integer}. This integer is the molecular weight, predicted from the amino acid sequence, of the individual connexin protein. For example, the molecular weight from the amino acid sequence for Cx32 was predicted to be 32 kDa (Note: the predicted molecular weight is different from the observed molecular weight of 27 kDa for this protein). Figure 2 illustrates the organization of a connexin protein within the plasma membrane (reproduced from Bruzzone et al. 1996b). The connexins are all integral membrane proteins with four transmembrane domains, labeled M1-M4, two extracellular loops, E1 & E2, and one intracellular loop (Bruzzone et al. 1996b). Both the N-terminus and the C-terminus reside in the cytosol.

Figure 1: This figure demonstrates the arrangement of connexin proteins to form a connexon (hemichannel) with a central pore. Reproduced from Bruzzone et al. 1996b.

Figure 2: This figure illustrates the conformation of connexin proteins in the plasma membrane. Connexin proteins have four membrane spanning regions, one intracellular loop, and 2 extracellular loops. Both the amine and carboxy termini reside in the cytosol. This figure was adapted from Bruzzone et al. 1996b.

Gap junctions in the brain

Direct evidence for the presence of gap junctions in the brain comes from electrotonic and dye coupling studies (for examples see: Dean et al. 1997, and Travagli et al. 1995; for review see: Dermietzel, and Spray, 1993). These data suggest that functional gap junctions are found within several nuclei and cell types throughout the brain. The presence of these channels was also determined indirectly by *in situ* hybridization, immunohistochemistry and Northern and Western blotting (for examples see: Nadarajah et al. 1996; and Naus et al. 1990). These studies identified the location and distribution of several connexin mRNA and proteins, including Cx32, Cx43, and Cx26. Each connexin protein appears to have a unique cellular expression and distribution within the brain. Cx43 is ubiquitously distributed throughout the brain and has been primarily localized to astrocytes, although it is expressed in other cell types including a small population of neurons in the visual cortex (Simburger et al. 1997). Cx32 and Cx26 are both found in neuronal populations (Micevych, and Abelson, 1991), although Cx32 is also expressed in oligodendrocytes (Dermietzel et al. 1997).

Plasticity of gap junctions?

Historically gap junctions were considered to be a primitive form of intercellular communication that allowed for passive exchange of molecules between adjacent cells. It is now apparent that gap junction-mediated communication is selective and that it can be dynamically controlled (Bruzzone et al. 1996a). A number of different factors are

believed to affect gap junction coupling including: pH, voltage, dopamine (DA), norepinephrine (NE), gamma-aminobutyric acid (GABA), acetylcholine (ACH), serotonin (5-HT), nitric oxide, second messengers, metabolites, DA agonists and antagonists, anesthetics ethanol, barbiturates and tumor promoters (see: Bennett et al. 1991, for review).

Interestingly, the diversity of the connexin proteins appears to allow for the formation of gap junction channels with distinct physiological properties, including conductances, permeability, and gating (Bevans, et al. 1997; Brink, et al. 1996; and Versalis, et al. 1994). It also appears that individual gap junction hemichannels and channels can be formed from more than one connexin isoform (Jiang, and Goodenough, 1996; and White, and Bruzzone, 1996). Connexons containing more than one connexin isoform are termed heteromeric (see Figure 3 reproduced from White, and Bruzzone, 1996). These hemichannels have different physiological properties than the hemichannels formed completely by either one of the connexins alone (homomeric hemichannel) (Wang, and Peracchia, 1998; Veenstra, 1996; and Brissette, et al. 1994). Complete gap junction channels can be comprised of two homomeric hemichannels made up of different connexin isoforms (Veenstra, 1996; and White, and Bruzzone, 1996). These channels are called heterotypic gap junctions (see Figure 3). However, the formation of heterotypic gap junctions is more controlled than previously anticipated (White, and Bruzzone, 1996). It is apparent that hemichannels comprised of a particular connexin isoform can only form complete channels with a subset of the other homomeric connexons (White, and Bruzzone, 1996). The compatibility and incompatibility of

particular connexons with other connexons is thought to provide a mechanism for compartmentalization or separation of communication pathways (White, and Bruzzone, 1996). In other words adjacent cells expressing different connexins may not be able to form gap junctions. This allows for selectivity in gap junction formation. It is predicted that alterations in gap junction gene expression could effect the formation of heteromeric hemichannels, and heterotypic channels and thus could alter both the physiological properties of gap junctions and the organization of communication pathways (White, and Bruzzone, 1996).

Recently, the D1/D2 receptor agonist, apomorphine, was shown to reorganize gap junction signaling pathways within the striatum. Onn, and Grace, 1994, found that the application of apomorphine to striatal slices resulted in the transfer of dye between groups of cells that were previously segregated. This suggests that dopamine and its agonists can alter coupling within this nucleus. The ability to reorganize signaling pathways within the brain in response to external stimuli may have biological consequences in physiological and pathological situations. However, the mechanism responsible for this change in intercellular coupling was not determined. It is possible that this change resulted from either dynamic changes in channel gating or plastic changes in gap junction distribution, type, and number. As discussed above it is predicted that alterations in gap junction gene expression might underlie changes in the organization of communication pathways.

Alterations in connexin gene expression have been demonstrated to allow cellular control over the selectivity of intercellular communication characteristics (Brissette et

al. 1994). Differences in unitary conductance and pore size have been found between gap junction channels comprised of different connexin species (Barrio et al. 1997; and Verselis et al. 1994). Brissette et al. 1994 demonstrated that a switch in connexin gene expression resulted in selective changes in channel permeability. Thus changes in the expression of an individual connexin gene have been demonstrated to effect the physiology of gap junction-mediated communication.

Cocaine self-administration

Bennett et al. 1998, demonstrated the first *in vivo* pharmacological modulation of connexin expression. Alterations of Cx32 gene expression were found in several regions of the brain, including the hippocampus, cortex, cerebellum, and striatum, following two weeks of cocaine self-administration. Some of the effects persisted for a period of 2-21 days. It is hypothesized that alterations in gap junction gene expression may be involved in the neuroadaptive mechanisms underlying drug addiction. An obvious question is whether other drugs of abuse with different mechanisms of action cause similar changes in Cx32 expression.

Figure 3: This figure illustrates the composition of heteromeric versus homomeric connexons and heterotypic versus homotypic gap junction channels. Reproduced from White, and Bruzzone, 1996.

A

Homomeric
connexon

Heteromeric
connexon

B

Homotypic
channel

Heterotypic
channel

Heteromeric
channel

Relevance to the physiology of opiates

The effect of opiate dependence and withdrawal on neuronal activity is well characterized (Liu et al. 1994; and Hayward et al. 1990). Opiate administration is known to hyperpolarize neurons and depress neurotransmitter release. On the other hand opiate withdrawal has been found to result in increased neuronal activity. However, the adaptive mechanisms that result in these changes in neuronal activity are only beginning to be determined.

Neurophysiological data suggest the possible involvement of gap junction communication in the neuroadaptive mechanisms underlying opiate dependence. Travagli et al. 1995, demonstrated that carbenoxolone blocked the hyperpolarization usually produced by opiate administration in locus coeruleus (LC) slices. This treatment is known to inhibit gap junction communication (Travagli et al. 1995), suggesting a role for gap junctions in mediating the effects of opiates within the LC. Neurons in the LC also exhibit a strong reaction to opiate withdrawal. However, a direct examination of the role of gap junctions in the withdrawal activity of LC neurons has not been done.

An extensive amount of research has focused on the role of the LC in both opiate dependence and withdrawal (see: Chrisitie et al. 1997 for review). Several researchers have proposed that the LC plays a principal and causal role in the expression of many withdrawal signs (Maldonado et al. 1992; and Hoshi et al. 1996); however chemical

lesions of the LC do not attenuate the withdrawal response (Chrisitie et al. 1997). This indicates that other regions may also be involved in the initiation and expression of the withdrawal syndrome. Several additional nuclei have been implicated in opiate withdrawal, including the periaqueductal grey (PAG), the paragigantocellularis (PGi), the raphe magnus, the rostral ventromedulla (RVM), the thalamus, the hypothalamus, and the nucleus accumbens (Maldonado et al. 1992; Hayward et al. 1990; Harris, and Aston-Jones, 1994; and Rasmussen, and Aghajanian, 1989). As well as being implicated in opiate withdrawal the nucleus accumbens has been thought to play a role in the reward process of drugs of abuse (Corrigall, and Vaccarino, 1988; and Wise et al. 1995). However, experimental evidence for the involvement of the nucleus accumbens in opiate dependence is inconsistent (Gerrits, and Van Ree, 1996). The involvement of gap junction-mediated intercellular communication within any of these regions, with regard to opiate dependence and withdrawal has not been examined.

The purpose of this thesis was two-fold. First we intended to characterize changes in Cx32 protein expression following heroin dependence and withdrawal. Pilot data indicated that there was a general decrease in the expression of Cx32 protein. Secondly, we hoped to determine whether these changes correlated with previously documented alterations in c-Fos expression.

Experiments

1. Western analysis of connexin 32 protein expression during heroin self-administration and withdrawal.

The first experiment was designed to identify regionally specific changes in the level of gap junction protein expression during heroin self-administration and withdrawal. Western blot analysis was used to survey brain regions for changes in the level of Cx32 protein expression.

Rationale

Localized modifications in neuronal activity are associated with both opiate dependence and the initiation and expression of the opiate withdrawal syndrome (Hayward et al. 1990; and Liu, J., 1994). The specific neuroadaptations that underlie these changes have not been completely elucidated; however, the modification of gap junction communication may play a role. Research conducted by Travagli et al. 1995, suggests that electrotonic coupling may mediate some aspects of opiate dependence and withdrawal.

Alterations in gap junction communication can have a profound effect on the physiology of a group of neurons (Travagli et al. 1995). To date, no examination of connexin expression during heroin self-administration and withdrawal has been conducted. Our hypothesis was that modification of connexin protein expression may

be involved in the adaptations of different nuclei to heroin self-administration and withdrawal. Specifically we intended to investigate nuclei within the medulla, thalamus, hypothalamus, and striatum for alterations in connexin 32 expression.

Of the fifteen connexins that have been isolated and cloned Cx32, Cx26, and Cx43 have been the most thoroughly examined within the CNS. Both Cx32 and Cx26 protein expression have been identified in several populations of neurons (Dermeitzel, and Spray, 1993; and Micevych, and Abelson, 1991). Plastic changes in the level of Cx32 expression have been previously demonstrated in response to cocaine self-administration (Bennett et al. 1998).

2. Immunolocalization of Cx32 and c-Fos expression during heroin self-administration and withdrawal

The primary purpose of the second experiment was to localize alterations in Cx32 expression to specific nuclei and cell types. This was to be accomplished by examining Cx32 immunoreactivity at selected time points during heroin withdrawal; control, 15-minutes, and 48 hours. The second purpose was to determine if modifications in gap junction expression during heroin dependence and withdrawal are correlated with alterations in c-Fos expression. c-Fos has previously been used as a marker of neuroadaptations during both heroin administration and withdrawal (Pontieri et al. 1997; Liu et al. 1994; and Hayward et al. 1990). In particular c-Fos expression has

been used to map regions of the brain involved in heroin withdrawal. In order to determine that changes in Cx32 IR were associated with previously documented neuroadaptations resulting from heroin self-administration and withdrawal we compared Cx32 immunoreactivity to c-Fos expression.

Rationale

Western analysis determines relative levels of protein between groups in a region; however, alterations in protein expression within particular nuclei may not be detected. Cx32 immunohistochemistry was used to localize alterations in connexin expression to specific nuclei within the regions originally screened by Western analysis.

Neuronal activation during opiate administration and withdrawal has been demonstrated to induce c-Fos expression (Hayward et al. 1990; and Liu, 1994). This has led to the use of c-Fos to map neuronal pathways and cell types activated during opiate dependence and withdrawal (Hayward et al. 1990; and Liu, 1994). Comparing the expression of Cx32 and c-Fos was to be used to determine if alterations in Cx32 are colocalized with the neuroadaptations associated with opiate administration and withdrawal.

Methods

Design

Animals

Male Wistar rats (N=36) 275-300g, were obtained from Charles River Laboratories, Quebec. Following quarantine the animals were housed in pairs in shoe box style polypropylene cages in a climate controlled vivarium (20°C). Twenty-eight of these animals were implanted with jugular cannulae and were provided access to heroin for a period of two weeks. The remaining animals (n=8) were used as age-matched controls.

Training

All rats were trained to press a response lever on an FR1 schedule for food reinforcement. During training the rats were placed on 23 hours of food deprivation in which animals had access to Purina food chow for 1 hour following operant training. The rats were placed into individual operant chambers and were presented with a response lever. Depression of the lever resulted in the delivery of a food pellet. The training consisted of the successful completion (>200 responses) of two 6 hour sessions.

Surgery

After training, 28 animals were anesthetized (60 mg kg⁻¹ pentobarbital) and chronically indwelling jugular cannulae were surgically implanted. Following surgery,

the rats were monitored until they became ambulatory. They were then housed in individual operant chambers for the duration of the experiment.

Self-administration

The rats were presented with a response lever, which provided access to heroin 24 hours a day on an FR1 schedule for a period of 2 weeks $\pm$ 2 days. Each response resulted in the infusion of heroin delivered at a dose of 25 $\mu\text{g kg}^{-1}$. No limit was set on the number of injections that could be taken in a daily session. Only data from animals that self-administered a minimum of 20 injections per day were included in the analysis.

Tissue

Western Analysis

Sixteen rats were anesthetized ($\sim$ 120 mg kg^{-1} pentobarbital, intraperitoneal (I.P.)) and then sacrificed by decapitation at various time points after their last infusion of heroin: 15 minutes (15-min), 24 hours (24-hr), 48 hours (48-hr) and one-week (1-week). Age-matched controls (N=4) were also sacrificed at this time. Medulla, thalamus, hypothalamus, accumbens, and dorsal striatum were dissected and flash frozen in dry-ice chilled iso-pentane.

A 1.5 mm coronal section was cut immediately anterior to the optic chiasm. Using the anterior commissure as a landmark we dissected the ventral (accumbens) and dorsal striatum. The rest of the tissue was then cut immediately anterior to the superior

colliculus. The diencephalon was separated from the overlying cortex and divided into the hypothalamus and thalamus. The cerebellum was separated and the medulla was defined as the region directly underlying the cerebellum.

Immunohistochemistry

Sixteen rats were perfused, with 4% paraformaldehyde including picric acid, 15 minutes (n=4) or 48 hours (n=4) after their last injection of heroin. Age-matched controls (n=4) were also perfused. Following 4 hours post-fixation the tissue was stored in 30% sucrose in 100 mM phosphate buffer (PB). Sections (20 µm and 40 µm thick) were cut on the sliding microtome and plates 11-16, 18-25, 26-31, and 32-39 (Paxinos, and Watson, 1982) were retained for further study. These plates encompass several nuclei implicated in opiate dependence and withdrawal including the nucleus accumbens; several areas within the hypothalamus; the thalamus; the amygdala; the periaqueductal grey; the locus coeruleus; the paraventricular nucleus; the rostromedial medulla; and the raphe magnus. The sections were then stored in cryoprotectant at -20°C for later analysis with floating immunohistochemistry.

Protein Extraction

Protein from the regions dissected for Western analysis was extracted using Trizol[®] according to the manufacturer's protocol (Life Sciences Technologies, Gibco-BRL). Briefly, the samples were homogenized in 1 ml Trizol[®] using a power homogenizer. The samples were then separated into three phases by adding 0.2 ml chloroform and subsequent centrifugation. The RNA remained exclusively in the upper phenol-

chloroform aqueous phase and was transferred to a fresh tube by pipette. The DNA was then precipitated out of the organic phase by the addition of 0.3 ml 100% ETOH and centrifugation. The remaining aqueous phase was transferred to a fresh tube for protein extraction. Isopropyl alcohol (1.5 ml) was added to the aqueous phase for every 1 ml Trizol[®] used in the initial homogenization. The protein pellet was obtained by centrifugation at 12,000 g for 10 minutes. This pellet was then washed 3 times in 2 ml of 300 mM guanidine hydrochloride in 95 % ETOH. Each wash consisted of a 20 minute incubation period and a 5 minute centrifugation period at 7500 g. After the final wash the pellet was vortexed in 2 ml ETOH. The pellets were then vacuumed dried for 5-10 minutes prior to solubilization. The pellet was redissolved in sodium dodecylsulfate (SDS) at 50°C. Insoluble material was sedimented by centrifugation at 10,000 g for 10 minutes. The supernatant was then transferred to a fresh tube and stored at -20°C for subsequent quantification and SDS-PAGE.

SDS-PAGE and Western Blot

The protein samples were separated according to molecular weight on a 12.5% polyacrylamide gel under reducing conditions using SDS-PAGE. Separated proteins were electroblotted from the gel to a nitrocellulose membrane. These membranes were then incubated with anti-Cx32 (Chemicon) specific to amino acids 95-125, localized within the cytoplasmic loop of the protein. Incubation of the membranes with anti- β -tubulin (Sigma-Aldrich) was used as a standard to ensure equal loading of protein samples. Secondary (Anti-mouse IgG 1:20,000, Sigma-Aldrich) and tertiary

(ExtrAviden 1:1000, Sigma-Aldrich) antibodies were used to amplify the signal and chemiluminescence was used to detect the reactions. Quantification of protein expression across the withdrawal time course was carried out using densitometry. Detailed summaries of SDS-PAGE and Western blotting techniques used can be found in Appendices A and B.

Densitometry

Autoradiographs obtained in Western analysis were placed on a light table with a camera affixed overhead. The camera sent information to a computer and monitor and the image was then digitized using MCID software. The densitometry program assigned Relative Optical Density values for each pixel within the selected field using the ROD function found in Appendix C. The mean of the ROD values for each pixel was then calculated and returned by the program as the ROD value for the selected field

$$\frac{\sum_x \sum_y ROD(I_{x,y})}{\Delta x \Delta y}; \text{ where } I_{xy} \text{ is the intensity of an individual pixel at location } xy.$$

It should be noted that the densitometry numbers obtained in this thesis are not quantitative but rather are a semi-quantitative estimate of relative intensities between samples within an autoradiograph.

Data analysis

In order to compare across autoradiographs relative optical density (ROD) numbers obtained from the densitometer were normalized, so that the mean of the observed

ROD scores equaled one. This was done to control for several concerns including the influence of exposure times and antibody binding. Without this procedure, the ROD scores would have been only indicative of relative intensities within an autoradiograph, and could not be used to compare between autoradiographs. If we wish to compare relative levels of connexin protein on different autoradiographs to each other, we must use some statistical procedure to control for exposure time and differential antibody binding. For instance one might derive the ratio of the obtained ROD values with respect to the control ROD value, setting the control ratio to one. However, this provides no estimate of the within-groups variability in the control population. Another alternative, adopted in the present analysis, is to compute the ratio of observed ROD to the mean ROD for each autoradiograph. This method is also biased since one is now assuming that the mean values do not vary across autoradiographs. However, the central limit theorem suggests that the mean values should show less variability than the control values would. Therefore all the densitometry values were normalized with respect to the mean of the autoradiographs.

The Cx32:tubulin index used was calculated by obtaining the ratio of normalized ROD values obtained above. This ratio was used to control for variations in the amount of protein loaded in different wells within a gel that would result in otherwise random variability in the ROD numbers. While not strictly applicable to the derived quantities thus obtained, analyses of variance (ANOVA) were performed on the Cx32:tubulin indices to get an indication of the significance level of the results obtained. Since using ratios as the arguments to the ANOVA (rather than direct measurables) tends to

overestimate the variability of the data, thereby reducing the likelihood of obtaining significant effects, it is hoped that this approach will be sufficiently conservative to get reliable results. We therefore term this analytic technique a semi-quantitative technique, and suggest that it provides a useful indicator of effects in the source data.

Immunohistochemistry

Cx32 (Chemicon) 1:1000 and c-Fos (Santa Cruz) 1:10,000 antibodies were examined individually using immunohistochemistry. The tissue was removed from the -20°C freezer and allowed to come to temperature in a 4°C fridge the day prior to staining. The next day the tissue was washed three times for 30 minutes in 100 mM phosphate buffer (PB) + 0.1% sodium azide. The sections were then incubated in 0.01% hydrogen peroxide (H₂O₂) in 10 mM PB for 20 minutes, to remove endogenous peroxidase. This was followed by three 5 minute washes in 10 mM PB + 0.1% sodium azide. Following the last of these washes the appropriate primary antibody was applied to the sections and they were left to incubate overnight at room temperature. The primary, secondary and tertiary antibodies were diluted to the appropriate concentration in a dilution solution. The secondary antibody Anti-mouse IgG (Sigma-Aldrich) was diluted 1:1000 and the tertiary antibody ExtrAviden (Sigma-Aldrich) was diluted 1:1000 for immunohistochemistry carried out for both Cx32 and c-Fos. The dilution solutions contained 5 ml of 10 mM PB, 1 ml of 2% bovine serum albumin and 1 ml of 2% λ-carageenan. The solution for the primary and secondary antibodies also contained 210 µl of 0.1% sodium azide. Each antibody application was followed by three 5 minute washes in 10 mM PB. On the second day the sections were incubated

for two hours each in secondary and tertiary antibodies sequentially. Following the last wash after the application of the tertiary antibody the sections were washed in 50 mM Tris-HCl containing 3,3' -diaminobenzidine (DAB) for 5 minutes. Nickel ammonium chloride (300 mg) was added to 50 ml of DAB solution used for reactions carried out in Cx32 immunohistochemistry. Nickel ammonium chloride was used to intensify the visualization of the Cx32 immunoreactive product. At the end of this wash 6 μ l of H₂O₂ was added to this solution. The sections were then mounted on to slides, dehydrated and coverslipped. To ensure even coverage of sections with antibodies and solutions all washes and reactions were carried out on a shaker table.

The sections that were examined for Rat IgG were incubated with a 1:400 dilution of anti-Rat IgG (Sigm-Aldrich) as if it were a primary antibody. The following day the sections were incubated with 1:1000 dilution of ExtrAviden. The sections were then reacted and prepared as discussed above.

Results

Dosing

The animals were placed into the study when they reached 20 inj day⁻¹. This took on average ~2 days. Self-administration was produced and maintained by a dose of 25µg kg⁻¹ inj⁻¹ of heroin. The average number of injections\24 hour session increased over the 2 week period from ~39 to ~83.

Westerns

Controls

In our Western blots the antibody recognized a 27 kDa band, consistent with the molecular weight previously reported for Cx32 protein (Paul, 1986). The anti-β-tubulin antibody recognized a 55 kDa band, consistent with the published molecular weight for β-tubulin (Weber, K., and Olson, M., 1985). Unidentified bands were frequently observed on the autoradiographs for both Cx32 and β-tubulin (Figure 4A and B). Secondary and tertiary antibodies in the absence of primary antibody reproduced the same pattern of nonspecific banding observed in the Cx32 and β-tubulin autoradiographs (Figure 4C). The molecular weights of these secondary and tertiary bands were determined to be approximately 39, 53, 69, 76, 84, 97, and 123 kDa. As illustrated in Figure 4 these nonspecific bands did not interfere with the detection of either the Cx32 or the β-tubulin bands. A detailed description of how the approximate molecular weights were obtained can be found in Appendix D.

Figure 4: A&B Representative Cx32 and β -tubulin autoradiographs. C. Nonspecific bands produced by incubation with the secondary and tertiary antibodies in the absence of primary antibody. Note that all the unidentified bands on the Cx32 and β -tubulin autoradiographs are accounted for by nonspecific binding of the secondary and tertiary antibodies.

β -Tubulin was used to indicate the relative loading of the protein samples. In order to ensure that β -tubulin was accurately reflecting the loading of the samples an independent measure of loading was used. A 12.5% polyacrylamide gel containing electrophoresed protein samples was stained using Commassie Blue (Figure 5A). The Commassie Blue stained gel demonstrated that the samples were evenly loaded. A second gel containing the same samples loaded using the same concentrations was electroblotted to membrane, and the membrane was then incubated with anti- β -tubulin (Figure 5B). The β -tubulin band also indicated that the gel was evenly loaded. Furthermore, β -tubulin was not affected by the treatment conditions.

Heroin

A visible decrease in Cx32 protein expression was observed within the medulla, in response to heroin self-administration and withdrawal (Figure 6A). This decrease was observed in the 15-min group and continued across the heroin withdrawal time course. The relative Cx32:tubulin indices were obtained using the method described above. The analysis of variance revealed significant differences across the time course in the medulla $F_{(4,12)} = 14.13$, $p < 0.001$. Scheffé *post hoc* tests were conducted to determine significant differences between pairs of groups. The 15-min, 24-hr, 48-hr, and 1-week groups were all observed to be significantly lower than control ($p < 0.005$) but were not significantly different from each other ($p > 0.05$). These data indicate that animals that self-administered heroin exhibited significant decreases in the Cx32:tubulin index within the medulla (see Figure 6B).

Figure 5: A. Commassie Blue stained gel loaded with series 4 samples from the medulla. B. β -tubulin autoradiograph of the same samples used in A. Note that the β -tubulin autoradiograph accurately represents the loading of the samples as reflected by Commassie Blue staining. Also the β -tubulin levels are unaffected by the treatment conditions.

A

C 15 24 48 1 week

B

C 15 24 48 1 week

Tubulin

Figure 6: A. A representative autoradiograph of Cx32 and β -tubulin expression in the medulla oblongata. B. Graph of the mean relative Cx32:tubulin indices for control (n=4), 15-min. (n=4), 24-hr (n=2), 48-hr (n=4), and 1-week (n=3) in the medulla. * denotes significant differences from control at $p < 0.005$

A

Cx32 Expression in Medulla

B

A visible decrease in Cx32 protein expression was also observed within the hypothalamus, in response to heroin self-administration and withdrawal (Figure 7A). A one-way ANOVA was performed to determine if there were significant differences between groups. Significant decreases in the relative Cx32:tubulin indices were evident across the time course within the hypothalamus $F_{(4,15)} = 10.33$, at $p < 0.001$. Scheffé *post hoc* tests were conducted to determine significant differences between pairs of groups. The 15 min. group was not significantly different from any other group, $p > 0.05$. The other experimental conditions (24-hr, 48-hr, and 1-week) were significantly different from control, $p < 0.005$ but not from each other, $p > 0.05$. These data indicate that withdrawal from heroin resulted in significant decreases in the Cx32:tubulin index within the hypothalamus (see Figure 7B).

Cx32 was observed to decrease at the 24 hour withdrawal time point within the dorsal striatum (Figure 8A). However the overall ANOVA of the relative Cx32:tubulin indices revealed no significant differences across the time course. The graph of the Cx32:tubulin index can be seen in Figure 8B.

No overall significant differences across groups in the relative Cx32:tubulin indices were observed in either the nucleus accumbens (Figure 9A & B), or the thalamus (Figure 10A & B). A summary of the relative Cx32:tubulin indices, ANOVA tables and Scheffé *post hoc* results for all regions can be found in Appendix D.

Figure 7: A representative autoradiograph of Cx32 and β -tubulin expression in the hypothalamus. B. Graph of the mean relative Cx32:tubulin indices for control (n=4), 15-min. (n=4), 24-hr (n=4), 48-hr (n=4), and 1-week (n=4) in the hypothalamus. * denotes significant differences from control indices at $p < 0.005$.

A

Cx32 Expression in Hypothalamus

B

Figure 8: A representative autoradiograph of Cx32 and β -tubulin expression in the dorsal striatum. B. Graph of the mean relative Cx32:tubulin indices for control (n=4), 15-min. (n=4), 24-hr (n=4), 48-hr (n=4), and 1-week (n=4) in the dorsal striatum. No significant differences were obtained.

A

Cx32 Expression in Dorsal Striatum

B

Figure 9: A representative autoradiograph of Cx32 and β -tubulin expression in the nucleus accumbens. B. Graph of the mean relative Cx32:tubulin indices for control (n=3), 15-min. (n=4), 24-hr (n=2), 48-hr (n=4), and 1-week (n=3) in the nucleus accumbens. No significant differences were obtained.

A

Cx32 Expression in Accumbens

B

Figure 10: A representative autoradiograph of Cx32 and β -tubulin expression in the thalamus. B. Graph of the mean relative Cx32:tubulin indices for control (n=4), 15-min. (n=4), 24-hr (n=3), 48-hr (n=4), and 1-week (n=4) in the thalamus. No significant differences were obtained.

A

Cx32 Expression in Thalamus

B

Immunohistochemistry

Cx32 IR

Inconsistent with the Western analysis no observable differences in Cx32 IR were found in any of the regions examined. In an attempt to optimize antibody concentrations and reaction times immunohistochemistry for all 12 animals was repeated three times. However, despite altering antibody concentrations and reaction times no differences were discernable. For example Figure 11 shows Cx32 IR within the hypothalamus for control, 15 min and 48 hour groups.

c-Fos IR

While increases were predicted c-Fos IR appeared to decrease during withdrawal in the hypothalamus (Figure 12) and periaqueductal grey (Figure 13).

Rat IgG

Increases in background immunoreactivity were consistently observed in the heroin self-administration tissue (15-min. group) and to a lesser extent in the heroin withdrawal tissue (48-hr group). Because of this result, the permeability of the blood brain barrier was examined. Increases in Rat IgG IR were observed in the brain parenchyma of both the 15-min. and 48-hr groups in comparison to control (Figure 14). Leakage of IgG was also observed around capillaries in the tissue from the 15-min. group (Figure 15).

Figure 11: Cx32 immunoreactivity (IR) in the paraventricular region of the hypothalamus, A. Control, B. 15-min., and C. 48-hr. No differences were observed in Cx32 IR.

Figure 12: This figure demonstrates c-Fos immunoreactivity (IR) in the hypothalamus. A. Control, B. 15-min. and C. 48-hr. There is an obvious decrease in c-Fos IR in both the 15-min. and 48 hr groups. Note also the increase in background staining in the 15-min. and 48-hr groups when compared to control.

Figure 13: This figure demonstrates c-Fos immunoreactivity (IR) in the periaqueductal grey in A. control, and B. 48-hr. c-Fos IR is decreased at the 48-hr time point in comparison to control. Again note the increase in background staining in the heroin withdrawal tissue.

Figure 14: Rat IgG immunoreactivity (IR) in the median eminence and hypothalamus, in A. control, B. 15-min., and C. 48-hr. Note the increase in Rat IgG IR in the brain parenchyma of the heroin treated tissue in comparison to control.

Figure 15: This figure illustrates the leakage of rat IgG from distinct capillaries in the 15-min. tissue. Note the uptake of IgG in the surrounding processes and cells.

Discussion

This thesis is the first report of alterations in the expression of the gap junction protein, Cx32, in response to opiate self-administration and withdrawal. The research adds to the recent observation in cocaine self-administration of changes in Cx32 protein expression (Bennett et al. 1998). Interestingly the effects of heroin and cocaine on Cx32 protein expression occurred in different regions and differed in time course indicating the pharmacological specificity of the observed changes.

The Western blot analyses demonstrated regionally specific long-term decreases in Cx32 protein across the withdrawal time course. We attempted to address the question of intracellular localization and cellular specificity through immunohistochemistry. Curiously, we were unable to demonstrate changes in Cx32 immunoreactivity (IR). We were, however, able to demonstrate differences in c-Fos IR. Also we unexpectedly observed extravasated IgG, indicative of blood brain barrier disturbance, in both the heroin self-administration and withdrawal tissue.

Western blot analysis

Consistent with the literature Cx32 protein was detected on autoradiographs as a 27 kDa band (Paul, 1986). The appearance of all extra bands on the autoradiographs were completely accounted for by nonspecific binding of the secondary and tertiary antibodies. The cytoskeleton marker β -tubulin was used to ensure that the protein samples were evenly loaded. Comparison of a Commassie Blue stained gel to a β -

tubulin autoradiograph, both loaded identically, indicated that β -tubulin was an accurate loading control. It also confirmed that β -tubulin was not affected by the treatment conditions. Densitometry provided a semi-quantitative and conservative estimate of the relative Cx32:tubulin indices.

Significant decreases in the Cx32:tubulin index were observed in both the hypothalamus and the medulla. In the hypothalamus significant decreases in the Cx32:tubulin index were observed at the 24-hr time point. This decrease persisted through the 1-week time point. Thus withdrawal from heroin resulted in long-term alterations in Cx32 expression within the hypothalamus.

In contrast significant decreases in the medulla of the Cx32:tubulin index were evident immediately following the final infusion of heroin. These decreases also remained at the 1-week time point. No significant differences within the withdrawal time course were observed. These results suggest that the alteration in Cx32 protein expression observed in the medulla was not dependent on withdrawal. Thus exposure to heroin alone may have resulted in the long-term neuroadaptive response observed in this region.

Interestingly no significant modifications in the Cx32:tubulin index were observed in either the thalamus, the nucleus accumbens, or the dorsal striatum. Although a perceptible decrease in Cx32 protein expression was observed at the 24-hr time point in

the dorsal striatum the overall ANOVA revealed no significant differences. From the above data it is evident that the effect on Cx32 protein expression is regionally specific.

The current project was designed with withdrawal in mind. We would expect that discontinuing access to heroin would have produced a short-term physiological (withdrawal) reaction. We had anticipated that we would observe an alteration during withdrawal and a slow return to control values across the time course. We therefore expected that there might be a short term (i.e. 1 -2 day effect) that would subside after a week. Instead we found that Cx32 protein expression was decreased on the last day of heroin exposure and remained suppressed at least until the 1-week time point when access to heroin was withdrawn. These data suggest that the changes in Cx32 expression may not be directly correlated with the time course of opiate withdrawal. However, no direct measures of withdrawal were taken and thus future studies should incorporate physiological measures of withdrawal (such as hypothermia, and/or diarrhea).

Cx32 Immunohistochemistry

The Western analysis identified long-term regionally specific decreases in Cx32 protein expression. We then sought to determine the specific nuclei, cell type and intracellular localization in which this decrease in Cx32 protein expression had occurred by using immunohistochemistry.

Abundant Cx32 IR was observed in all the regions examined; however, we were unable to identify changes in Cx32 IR in either the hypothalamus or the medulla. The pattern of Cx32 IR within specific nuclei throughout the brain was consistent with the literature (Yamamoto et al. 1989; Nadarajah et al. 1996; and Li, Hertzberg, and Nagy, 1997). Nevertheless, it should be noted that the literature presents a diverse picture with respect to the identification of different cell types and intracellular localization of Cx32 IR products (Yamamoto et al. 1989; Yamamoto, Hertzberg, and Nagy, 1990; Nadarajah et al. 1996; and Li, Hertzberg, and Nagy, 1997). It appears that the extent of Cx32 IR in a particular cell type and its intracellular location varies with both the epitope the antibody is directed and the duration and type of fixation (Yamamoto et al. 1989). Thus it is possible that a different fixation process or antibody might have produced different immunohistochemical results.

It was apparent from the literature that Cx32 IR, within the cell, can be detected outside of functional gap junctions (Yamamoto et al. 1989; Yamamoto, Hertzberg, and Nagy, 1990; Nadarajah et al. 1996; and Li, Hertzberg, and Nagy, 1997). The determination of the intracellular location of Cx32 IR was then important to identifying whether the decrease in Cx32 protein was associated with the membrane. The use of nickel intensification in the current immunohistochemical study resulted in large purple/black immunoreactive products that often filled the entire cell body. Thus we were unable to determine the intracellular localization of the Cx32 IR. However, it is not unusual for light microscopy studies to fail to definitively determine the

intracellular localization of Cx32 IR (Yamamoto, Hertzberg, and Nagy, 1990). Often electron microscopy is necessary for such a determination (Yamamoto, Hertzberg, and Nagy, 1990).

Blood Brain Barrier Dysfunction

Higher background staining was consistently observed in the heroin self-administration and heroin withdrawal tissue when compared to control. It was hypothesized that this may have resulted from the extravasation of IgG resulting from blood brain barrier compromise. Anti-Rat IgG IR is often used as an indicator of BBB dysfunction (Sokrab et al. 1988; Schmidt-Kastner et al. 1993; Moss, and Hoyer, 1996). Extravasation of rat IgG was observed in the parenchyma and around a few discrete capillaries within the tissue from both the 15-min and the 48-hr group. The exudated IgG was taken up into a few cells within the hippocampus, occipital cortex, and by cells adjacent to the capillaries in the hypothalamus. The observation of mild BBB dysfunction in heroin and heroin withdrawal tissue is a novel finding and warrants further investigation.

Endogenous rat IgG in tissue can crossreact with bridge antibodies directed at mouse IgG, like the one used in this immunohistochemical study (Schmidt-Kastner et al. 1993). This can lead to nonspecific binding of secondary and tertiary antibodies. Consequently, false-positive staining might help explain the failure of the immunohistochemistry to confirm decreases in Cx32 protein in either the medulla or

the hypothalamus observed by Western blot analysis.

c-Fos Immunohistochemistry

Decreases in c-Fos IR during heroin self-administration and withdrawal were observed in both the periaqueductal grey and the periventricular hypothalamus. This result is contrary to several publications that have reported increases in c-Fos expression in response to opiate withdrawal (Beckmann, Matsumoto, and Wilce, 1995; Liu, Nickolenko, and Sharp, 1994; Chieng, Keay, and Christie, 1995; Hayward, Duman, and Nestler, 1990; Chang, Squinto, and Harlan, 1988). However, the literature cited used precipitated withdrawal in contrast with the current study's use of abstinence as a model of withdrawal. Precipitated withdrawal involves the challenge of opiate dependent animals with opiate antagonists. The precipitated withdrawal syndrome may be more severe, and is strongly characterized by a sudden transition to a withdrawal state. The abstinence withdrawal occurs over several days whereas the precipitated withdrawal process occurs within hours. The current data may simply indicate that the time course of c-Fos changes is different for the two forms of withdrawal. However, this could also indicate that the biochemical markers of precipitated withdrawal are different from those involved in abstinence withdrawal and thus merits further study.

Cx32 Protein Expression and Intercellular Coupling

It should be re-emphasized that this is the first demonstration of a modulation of gap junction protein expression following long term heroin exposure. However, it is premature in the investigation to conclude whether these changes are functionally significant. We can only speculate as to the possible consequences of the observed decrease on Cx32 expression.

There are at least three mechanisms by which decreases in Cx32 protein could affect gap junction communication.

1. The decreases in Cx32 protein observed in the medulla and hypothalamus could represent decreases in intercellular coupling. A study by Laird et al. 1995, determined the half-life of gap junctional plaques in mammary cells by blocking transport of *de novo* formed connexin proteins to the membrane with Brefeldin A (BFA). The preexisting gap junctional plaques were lost following six hours of treatment with BFA. Since heroin self-administration and withdrawal resulted in significant decrease in Cx32 protein that persisted even at the 1 week time point it might reasonably be expected that decreases in Cx32 gap junctions are occurring.
2. The second hypothesis is that the observed decrease in Cx32 protein results in a change in channel composition, perhaps a switch from heteromeric to homomeric hemichannels. It appears that different connexin isomers can be co-expressed in the same cell both *in vitro* (White, and Bruzzone, 1996; Bevans et al. 1998; and Wang, and Peracchia, 1998) and *in vivo* (Jiang, and Goodenough, 1996). These cells are

then capable of forming heteromeric connexons, a combination of more than one connexin isoform in the same hemichannel structure. As mentioned in the introduction the composition of a connexon influences the physiological properties of the channel including the conductance, permeability and gating (Wang, and Peracchia, 1998; Bevens et al. 1998; Brink, 1996; Veenstra, 1996; Brissette et al. 1994; and Verselis et al. 1994). The presence of heteromeric connexons then could result in new regulatory properties for gap junctions (Bevens et al. 1998). Of the 15 connexin proteins that have been cloned to date only a small subset has been examined in brain (Cx26, Cx32, Cx43, Cx37, Cx31 and Cx45). Cx45 coexists with Cx32 in oligodendrocytes (Dermietzel et al. 1997). Whether other connexins coexist with Cx32 in neurons has not yet been determined. Bevens et al. (1998), suggested that cells that co-express more than one connexin may modify the physiology of their gap junction channels by altering the relative expression levels of the different connexin isoforms. Under our control conditions cells may contain heteromeric connexons, however, with the loss of one of the proteins, in this case Cx32, these hemichannels may become homomeric connexons. These homomeric connexons would have different channel properties than the original heteromeric hemichannel. Consequently a decrease in the expression of one connexin could alter communication characteristics of gap junction channels.

3. The final alternative also results in an alteration in channel composition, in this case a switch in connexin gene being expressed. It may be possible that heroin effects the expression of more than one connexin. In this case we may see a decrease in

one connexin and an increase in another. Since each connexin isomer forms channels with specific conductances, pore permeabilities and gating properties a switch in connexin gene expression would alter the physiological properties of the channel (Saez et al. 1991). For instance the switch in gene expression then could alter the selectivity between second messengers that are capable of being passed between coupled cells (Bevans et al. 1998; and White, and Bruzzone, 1996). Such a switch in connexin gene expression could also alter communication pathways. Different connexin proteins can form full gap junction channels with only a subset of the other connexon hemichannels (White, and Bruzzone, 1996). This exclusionary process results in the formation of specific communication pathways (White, and Bruzzone, 1996). A switch in connexin gene expression may allow cells to communicate with cells that were previously incompatible. The altering of communication pathways has been observed *in vitro* in striatal slices following administration of apomorphine (Onn, and Grace, 1994). Thus a switch in connexin gene expression could result in altering both physiological properties of gap junction channels and communication pathways.

Therefore it is possible that the observed decrease in Cx32 protein expression could represent either a change in the overall number of gap junctions or a change in the composition of gap junction channels. In order to determine the functional significance of the observed changes a number of future studies need to address the following questions: Are the decreases in Cx32 protein associated with the membrane? In what

cell types are the decreases occurring? Do these cells express any other connexins? Is the expression of other connexins altered by heroin self-administration and withdrawal? What is happening to intercellular coupling?

In conclusion we have demonstrated regionally specific changes in Cx32 protein expression following heroin self-administration. These results could be used to direct future investigations into links with reinforcement and withdrawal.

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Appendix A

SDS-PAGE

Background

Laemmli originally developed SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 1970. It is one of the most widely used techniques for the analysis of protein samples. This technique involves the reaction of proteins with the anionic detergent sodium dodecylsulfate (SDS). The SDS binds to the proteins and disrupts all noncovalent bonds causing the macromolecules to unfold (Garfin, 1990). This results in the formation of long negatively charged polypeptide complexes (Garfin, 1990). Often 2-mercaptoethanol is also added to the sample. 2-mercaptoethanol further denatures the proteins breaking them down into their constituent subunits (Garfin, 1990).

Almost all proteins bind a similar amount of SDS per unit weight of protein, approximately 1.4 mg of SDS/ 1 mg protein (Garfin, 1990). Thus the length of the SDS-polypeptide is roughly proportional to the molecular weight of the protein. These complexes can then separate according to molecular weight during electrophoresis. The migration of the SDS-polypeptide complexes along the gel is inversely proportional to the logarithm of the molecular weight of the proteins (Garfin, 1990).

The use of a discontinuous gel system improves the resolution of the samples (Garfin, 1990). This system consists of two gels a stacking gel and a separating gel. The stacking gel is cast on top of the separating gel. The two gels are cast with different pore sizes, pH, and ionic strength. When an electric field is applied the ions

begin to move toward the anode. Within the stacking gel the samples become sandwiched between fast moving chloride ions (present throughout the system) and slow moving glycinate ions (present in the electrophoresis buffer); resulting in the concentration of the samples (Garfin, 1990). When this reaches the separating gel the mobilities of the SDS-polypeptide complexes are changed and the glycine ions overtake them (Garfin, 1990). This leaves the protein complexes to separate from one another according to size within the separating gel matrix.

The polyacrylamide gels are formed from the polymerization of an acrylamide monomer and a crosslinker, bis acrylamide (Garfin, 1990). The addition of ammonium persulfate and TEMED to the acrylamide solution initiates the polymerization reaction. Ammonium persulfate activates TEMED and leaves it with an unpaired electron (Garfin, 1990). This free radical then reacts with several acrylamide monomers to form a polymer. The bis acrylamide is incorporated into the polymer and forms crosslinks between them. Since oxygen is a radical scavenger, and thus interferes with polymerization, the solutions must be deaerated prior to the addition of ammonium persulfate and TEMED (Garfin, 1990).

The pore size of the gel is essentially the resistance the gel imparts to the migration of the protein molecules (Garfin, 1990). Altering the % of total acrylamide (% T) and the ratio of the crosslinking agent (bis acrylamide) to acrylamide monomer (% C) can adjust the pore size. These values may be determined for the best resolution of proteins of different molecular weights. Smaller pore gels result from increasing the %T and adjusting the %C and are used to separate smaller molecular weight proteins. Whereas

larger pore gels separate large molecular weight proteins and result from decreasing the %T.

Technique (adapted from a protocol presented by S. R. Gallagher, and J. A. Smith in Short Protocols in Molecular Biology, 1992)

Used minigel apparatus (8.5 cm x 4.5 cm)

1. The casting apparatus was assembled according to the specifications provided by the manufacturer (Bio-Rad). The separating gel was then prepared as follows:

6.25 ml of 30% acrylamide/.8% bisacrylamide
3.75 ml of 4x Tris-Cl/SDS pH 8.8
5.0 ml of dH₂O

The solution was deaerated and then the ammonium persulfate and TEMED were added.

0.05 ml of 10% ammonium persulfate
0.01 ml of TEMED

The separating gel solution was then carefully pipetted into the narrow chamber formed by the two glass plates and spacers. The solution was poured to a level about 1 cm below where the bottom of the well-forming comb would come when it was in position. This solution was then overlaid with H₂O saturated isobutanol.

The gel was allowed to polymerize for 0.5 to 1.5 hours.

2. The stacking gel was prepared as follows:

0.65 ml of 30% acrylamide/.8% bisacrylamide
1.25 ml of 4x Tris-HCl/SDS pH 6.8

3.05 ml of dH₂O

The solution was degassed and then ammonium persulfate and TEMED were added.

0.025 ml of 10% ammonium persulfate
0.005 ml of TEMED

The isobutanol was poured off of the separating gel and the top was rinsed with 1 X Tris-Cl/SDS pH 8.8. Then the stacking gel was pipetted into the remaining space in the chamber. The well-forming comb was then inserted and the gel was allowed to set (approximately 0.5-1 hour).

3. After the stacking gel polymerized the comb was removed. The casting stand was then dismantled and the clamps holding the gels were placed in the electrophoresis reservoir. The 5 X electrophoresis buffer was diluted to 1 X and poured into the upper chamber of the reservoir.
4. The protein samples were prepared while the stacking gel was setting. They were diluted 1:1 in 2 X sample loading buffer. 2-mercaptoethanol was added and samples were then heated in boiling water for 5 minutes.
5. The samples were then loaded into the gel by carefully pipetting each sample into a sample well. When all the samples were loaded the remaining electrophoresis buffer was poured into the lower chamber and the electrophoresis was started. An applied voltage of 120 V was used.
6. Electrophoresis continued until the bromophenol blue dye front reached the bottom

of the glass plates. The gels were then removed from the glass plates and placed into Towbin transfer buffer for two 10-minute washes prior to electroblotting onto nitrocellulose membrane.

7. Prior to the transfer two nitrocellulose membranes and 8 pieces of filter paper were cut to the same dimensions as the two gels. These were then soaked in the transfer buffer for a period of 30 minutes.
8. For each gel/membrane sandwich two pieces of filter paper were placed on the bottom, followed immediately by the membrane, then the gel, and finally two more pieces of filter paper. The transfer was carried out on a semi-dry transfer unit (Bio-Rad) with a current limit of 30 mA and a voltage of 24 V for a period of 30-45 minutes.
9. Following the transfer the membranes were stained in Ponceau S. Proteins bind covalently to the nitrocellulose membrane; these membrane-bound proteins are then available for analysis using Western blotting.

5X SDS/electrophoresis buffer
15.1 g Tris base
72 g glycine
5.0 g SDS
H₂O to 1000 ml
Dilute to 1X before use (pH 8.3)

2X SDS/sample buffer
25 ml Tris-Cl/SDS, pH 6.8
20 ml glycerol
4 g SDS
1mg bromophenol blue
100 ml H₂O

Appendix B

Western blotting

Western blotting allows for the visualization of individual protein bands by the detection of specific protein-antibody interactions. Membranes containing electrophoretically separated proteins are incubated with a primary antibody specific to the protein of interest. The signal intensity is increased by the application of secondary and tertiary antibodies. The immunoreactive bands can be visualized by several reactions, such as horse-radish peroxidase or chemiluminescence. Comparisons of the relative intensity of immunoreactive bands can be made between different samples run on the same gel. Antibodies specific to cytoskeletal markers, such as Tubulin, are used to determine that the loading of the samples in SDS-PAGE was even.

Technique

The Ponceau S stain was washed off the nitrocellulose membranes by two 10 minute washes in 10 mM phosphate buffered saline (PBS) pH 7.5. To prevent nonspecific binding the membranes were blocked for 1 hour in 10 mM PBS pH 7.5 containing 1% heat-denatured casein. The membranes were then incubated overnight at 4°C with the primary antibody, anti-Cx32, diluted (1:1000) in the same blocking solution. Following the incubation with the primary antibody the membranes were washed twice in 10 mM PBS for 10 minutes. The membranes were also incubated with the secondary (Anti-mouse IgG, Sigma-Aldrich) and tertiary (ExtrAviden, Sigma-Aldrich) antibodies. These antibodies were diluted, 1:20,000 and 1:1000 respectively, in the blocking

solution. Each antibody was incubated with the membrane for 1 hour and the incubations were followed by washes in 10 mM PBS. Immunoreactive bands were visualized using chemiluminescence according to the protocol provided by the manufacturer (Boehringer Mannheim).

Appendix C

Densitometry Function

The densitometer program used a predetermined set of values or Relative Optical Density units corresponding to a standard gray scale. The ROD units used by the program can be found in column 2 of Table C1. In order to approximate the function used by the program the ROD units were plotted against an arbitrary but increasing scale of relative blackness or intensity (column 1 Table C1). A logarithmic curve fit yielding the function and line graph represented in Figure C1 was performed. The fitness of the function can be viewed by comparing the values returned by the function (column 3 of Table C1) to those given by the program (column 2 of Table C1). By plotting the function we can determine the linear range of the program.

Table C1

Relative Blackness	Relative Optical Density	ROD Function
0.5	0.02	0.0179
1.5	0.06	0.0596
2.5	0.10	0.1030
3.5	0.15	0.1485
4.5	0.20	0.2013
5.5	0.27	0.2652
6.5	0.34	0.3406
7.5	0.42	0.4267
8.5	0.53	0.5279
9.5	0.67	0.6642
10.5	0.88	0.8851
11.5	1.29	1.2888

Figure C1

Using ROD Function: $1.24691e-3 + 2.74175e-2t + 1.49406e-2t^3 - 6.8375e-3t^3 + 1.54047e-3t^4 - 1.4t^5 + 5.68773e-6t^6$

Appendix D

Determination of the Molecular Weights of Autoradiographic Bands

The molecular weights (MW) of the bands observed on the autoradiographs were determined by plotting the $\log_{10}$ MW of the standard bands versus the distance they traveled (measured in cm) from the top of the separating gel. A logarithmic curve fit $y = A \ln B + C$ was performed for both the secondary and tertiary, and Cx32 and β -tubulin autoradiographs, yielding two separate curves with an $R^2 = 0.9975$ and $R^2 = 0.9952$ respectively. The curves are presented in Figure D1 (Secondary and Tertiary) and Figure D2 (Cx32 and β -tubulin). The equations were then used to determine the $\log_{10}$ MW for the distances traveled by the bands of interest. Then the anti-log of that value was obtained to yield the approximate molecular weight. The MW of the standards, the distance traveled, the predicted MW, the error, and the predicted MW for secondary and tertiary and Cx32 and β -tubulin can be seen in Tables D1 and D2.

Table D1

Molecular Weight Standards	Log MW	Distance Travelled (cm)	Predicted Log Mol. Wt.	Predicted Mol. Wt.	Error
116.25	2.065392962	1	2.0701	117.5	1.3
97.4	1.988558957	1.25	1.972095352	93.8	-3.6
66.2	1.820857989	1.7	1.837048072	68.7	2.5
45	1.653212514	2.6	1.650439373	44.7	-0.3
31	1.491361694	3.75	1.489584835	30.9	-0.1

Secondary and Tertiary Bands

3.05	1.580329813	38.0
2.2	1.723809527	52.9
0.95	2.092628015	123.8
1.2	1.990024372	97.7
1.4	1.922321394	83.6
1.55	1.877618434	75.4
1.7	1.8370480	68.7

Table D2

Molecular Weight Standards	Log MW	Distance Travelled (cm)	Predicted Log Mol. Wt.	Predicted Mol. Wt.	Error
116.25	2.065392962	0.6	2.077707314	119.59343	3.3
97.4	1.988558957	0.8	1.961915279	91.604177	-5.8
66.2	1.820857989	1.1	1.833737653	68.192663	2.0
45	1.653212514	1.7	1.658522129	45.55354	0.6
31	1.491361694	2.6	1.487506643	30.726044	-0.3
			Cx32 Band		
		3	1.429908554	26.909681	
			Tubulin		
		1.4	1.736669925	54.534323	

Figure D1

Secondary and Tertiary Bands

Figure D2

Tubulin and Cx32 bands

Appendix E

Summary of Cx32:tubulin indices, ANOVA summary tables and Scheffé *post hoc* tests

The Tables are presented in the following order: Table E1 medulla, Table E2

hypothalamus, Table E3 dorsal striatum, Table E4 thalamus, and Table E5 accumbens.

Table E1: Medulla

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Control	4	10.20022	2.550055	0.433247
15 min.	4	0.98463	0.246158	0.103693
24 hours	2	0.556599	0.2783	0.154901
48 hours	4	2.852531	0.713133	0.241792
1 week	3	1.590674	0.530225	0.228012

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	13.87763	4	3.469406	14.12662	0.000172	3.259160053
Within Groups	2.947123	12	0.245594			
Total	16.82475	16				

Scheffe F Crit.

P	F Crit.
0.05	13.03664
0.01	21.64779
0.005	26.08431
0.001	38.53347

Scheffe Value Control vs.

15 min.	24 hours	48 hours	1 week
43.22542	28.01849	27.47858	28.47711

**Scheffe Value 48
hours. vs.**

15 min.	24 hours	48 hours	1 week
1.775828	1.026519	0	0.233524

Table E2: Hypothalamus

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Control	4	7.995	1.99875	0.242636
15 min.	4	4.268	1.067	0.133225
24 hours	4	2.406	0.6015	0.037466
48 hours	4	1.608	0.402	0.089805
1 week	4	2.532	0.633	0.287727

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	6.541419	4	1.6353548	10.33912	0.000318	3.055568243
Within Groups	2.372574	15	0.1581716			
Total	8.913993	19				

Scheffe F Crit.

P	F Crit.
0.05	12.222273
0.01	19.572781
0.005	23.211669
0.001	33.01102

Scheffe Value Control vs.

15 min.	24 hours	48 hours	1 week
10.97742	24.68595	32.238541	23.58544

Scheffe Value 15 min. vs.

24 hours 48 hours 1 week
 2.739939 5.5917124 2.381667

Table E3: Dorsal Striatum

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Control	4	4.250117	1.062529	0.230915
15 min.	4	4.755379	1.188845	0.189867
24 hours	4	0.568397	0.142099	0.079949
48 hours	4	7.534661	1.883665	1.290591
1 week	4	5.564607	1.391152	1.43551

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	6.480431	4	1.620108	2.510369	0.085822992	3.055568243
Within Groups	9.680496	15	0.645366			
Total	16.16093	19				

Table E4: Thalamus

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Control	4	4.016093	1.004023	0.275315
15 min.	4	3.821448	0.955362	1.655523
24 hours	3	3.62364	1.20788	0.211702
48 hours	4	4.387254	1.096814	0.281035
1 week	4	4.877955	1.219489	0.477173

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	0.211005	4	0.052751	0.086981	0.985062	3.112248237
Within Groups	8.490544	14	0.606467			
Total	8.70155	18				

Table E5: Accumbens

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Control	3	2.745543	0.915181	0.021852
15 min.	4	3.880275	0.970069	0.080197
24 hours	2	2.777263	1.388632	0.229317
48 hours	4	4.203168	1.050792	0.101444
1 week	3	2.649373	0.883124	0.107781

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	0.37445	4	0.093613	0.996353	0.449677	3.356689149
Within Groups	1.033508	11	0.093955			
Total	1.407958	15				

IMAGE EVALUATION TEST TARGET (QA-3)

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