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Sensitivity to Dopamine D1/D2 Receptor Stimulation in Mice Lacking Connexin-32 or Connexin-36

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SENSITIVITY TO DOPAMINE D1/D2 RECEPTOR STIMULATION IN MICE LACKING
CONNEXIN-32 OR CONNEXIN-36

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
The Applied Biopsychology Program

by

James O. McKenna III

B.S., Tulane University, 2000

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Acknowledgements

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Abstract

Previous work has shown D1/D2 requisite synergism can still occur in the striatum in the absence of action potentials. Some nonclassical communication such as gap junctions may be allowing the segregated dopamine (DA) receptors to interact to produce stereotyped motor activity. Connexin-32 (Cx32) and connexin-36 (Cx36) were targeted for study due to their abundance in neural tissues and presence in the striatum. Mice lacking either the Cx32 or Cx36 gene and their respective wild-type littermates were compared on a climbing behavior task used to gauge their dopaminergic activity after receiving either saline, D1 agonist, D2 agonist, or both D1 and D2 agonists. The results showed that D1/D2 requisite synergism was still intact in both strains of mice. The Cx32 WT mice displayed significantly greater scores than the KO mice in the D1/D2 treatment. The Cx36 mice did not display a significant genotype difference, but a trend was observed with the KO females having larger scores relative to WT females or to males of either genotype.

Introduction

Dopamine (DA) is a neurotransmitter that became a topic of research when it was first discovered in 1959. The highest concentrations of DA are found in the brain, specifically in the basal ganglia, midbrain, and hypothalamic regions (Carlsson, 1959). Since its discovery, a broad array of functions has been related to DA including movement, motivation, attention, reinforcement, and cognition (LaHoste and Marshall, 1996). Disruptions in the DA system play a role in the development of many perplexing human ailments. Disorders such as schizophrenia, Parkinson's disease, and attention-deficit/hyperactivity disorder all show an abnormality regarding DA function (Majovski et al., 1981; LaHoste et al., 1996).

Dopamine's role in motor control has been examined for many years due to its well-documented depletion in Parkinsonian patients, who display abnormal motor behaviors, such as, tremor, rigidity, akinesia, or postural abnormalities (Maier-Hoehn, 1992). Those with Parkinson's disease become DA-deficient in the striatum, the main input structure of the basal ganglia, where 80% of the brain's DA is found. The deficiency was found to be related to a gradual loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Hornykiewicz, 1966). The striatum and SNpc are both crucial structures within the basal ganglia that influence movement.

Dopaminergic pathways

There are two major dopaminergic pathways that follow a dorsal or ventral mesotelencephalic route. The ventral or mesocorticolimbic pathway, originating in the ventral

tegmental area (VTA; A10), terminates predominantly in the nucleus accumbens, olfactory

tubercles, medial caudate-putamen, and prefrontal cortex (Fuxe et al., 1985).

The DA projections to the nucleus accumbens are strongly implicated in having a role in

the addictive properties of drugs of abuse (Bozarth and Wise, 1983; Kuhar et al., 1991) as well as

in reinforcement in general (Wise and Rompre, 1989). The dorsal or nigrostriatal pathway is the

most pronounced dopaminergic pathway in the brain, and its normal functioning is essential for

motor control (Hornykiewicz, 1973; Feldman et al., 1997). The nigrostriatal pathway is

composed of dopaminergic fibers originating in the SNpc, that project primarily to the caudate

and putamen (Fuxe et al., 1985). The neuronal makeup of the structures in this nigrostriatal

pathway provides clues to understanding the pathway's function.

Basal Ganglia

The basal ganglia are made up of a group of brain structures that affect behavior,

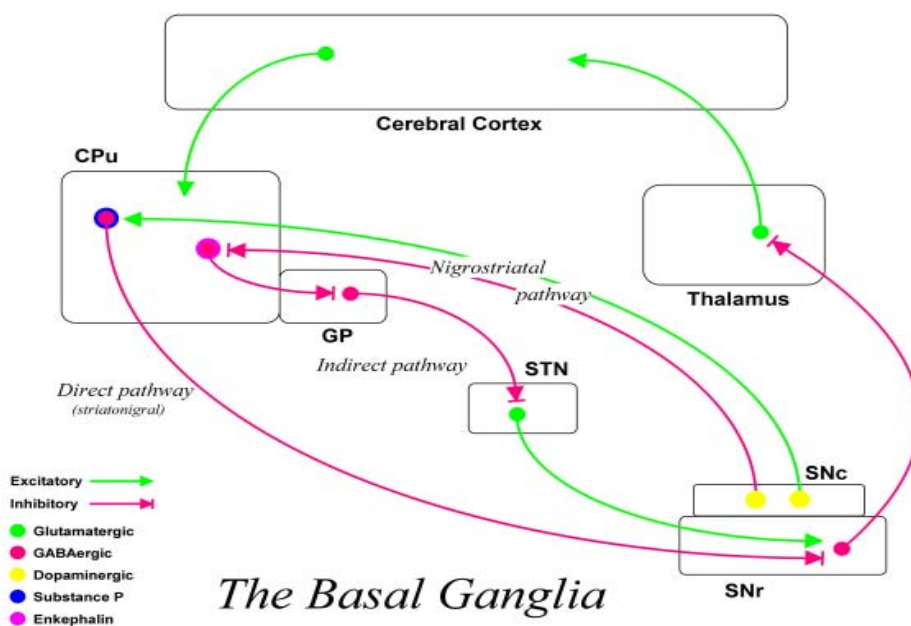especially voluntary movement (Fig. 1). The striatum is the main structure within the basal

Figure 1. Schematic representation of the basal ganglia. Dopaminergic neurons of the SNc have either inhibitory or excitatory effects on striatal projection neurons depending on the postsynaptic DA receptor. The main output structure of the basal ganglia is the SNr, which facilitates behavior when it is inhibited.

ganglia, and it has three components: caudate, putamen, and nucleus accumbens. (In rodents the caudate and putamen are fused and therefore referred to as the caudate-putamen (CPu)). About 90-95% of the striatum is made of GABA-ergic medium spiny neurons whose axons project outside of the striatum. The remaining 5-10% of striatal neurons consists of large aspiny cholinergic and medium-size GABA-ergic interneurons whose axons do not exit the striatum (Gerfen, 1992). Other structures of the basal ganglia include the globus pallidus (GP), the substantia nigra pars reticulata (SNr) and the subthalamic nucleus.

Through its connections with other brain structures, the basal ganglia form a loop that modulates cortical activity. The main input to the basal ganglia comes from the cerebral cortex, and the basal ganglia's main output is to the cerebral cortex via the thalamus (Herrero et al., 2002).

Multiple dopamine receptors

All DA receptors use guanine nucleotide binding proteins (G-proteins) to effect change intracellularly. In most cases, adenylyl cyclase (AC) is the second messenger involved in DA receptor activation. Kebabian and Calne discovered that two different families of DA receptors (D1, D2) existed. The D1 family of receptors is coupled with stimulatory G-proteins (G_s) that stimulate AC activity, while the D2 family of receptors is coupled with inhibitory G-proteins (G_i) to inhibit AC (Kebabian and Calne, 1979). Further study, using new cloning technology, has revealed the D1 family includes D_1 and D_5 sub-type receptors, and the D2 family includes D_2, D_3, and D_4 sub-type receptors. (The subscript numbers are used to indicate the specific type of DA receptor, while the normal type numbers indicate the family). The D_1 and D_2 sub-type receptors are found in much greater abundance in the brain than the D_3, D_4, or D_5 sub-types

(Sibley and Monsma, 1992). The downstream actions of these receptors are still being theorized and researched.

D1/D2 requisite synergism

The D1 and D2 receptor families both serve an important function in regards to motor behavior. Dose dependent effects on motor behavior can be observed after stimulation of DA receptors with indirect agonists (e.g., amphetamine), or with post-synaptically acting agonists that do not distinguish between D1 and D2 receptors (e.g., apomorphine). Lower doses of these agonists elicit locomotion, whereas higher doses elicit species-specific patterns of stereotyped motor behavior (e.g., intense sniffing in rats). Although not originally appreciated, it is now known that in normal rats co-activation of D1 and D2 receptors is required to elicit locomotion and motor stereotypy, which is a phenomenon now referred to as D1/D2 requisite synergism (Walters et al., 1987; White et al., 1988; LaHoste and Marshall, 1993). Earlier studies showing locomotion and stereotypy following administration of an exogenous D2 agonist did not account for endogenous DA, which stimulates D1 receptors. Such effects disappear when the endogenous DA is depleted by α-methyl-para-tyrosine (AMPT) (Braun and Chase, 1986) or by blockade of D1 receptors (LaHoste and Marshall, 1992). Furthermore, the motor activating effects of a mixed D1/D2 agonist, like apomorphine, can be completely blocked by prior administration of either a selective D1 or D2 antagonist (Lewis et al., 1983; Mailman et al., 1984). This remarkable D1/D2 synergism is not restricted to the behavior of rats; it is observed in every species studied, including mice, the species of choice for genetic engineering.

Mice respond to DA agonists with particular motor behaviors. A high dose of apomorphine will induce stereotyped cage climbing behavior in mice when they are placed in a wire mesh cylindrical cage. The mice will spend the majority of their time with all four paws on

the apparatus, rather than merely running up and down (Protais et al., 1976; Costall et al., 1978). This effect can be blocked by a selective D1 or D2 antagonist (Iorio et al., 1983; Moore and Axton, 1988). Cage climbing behavior can also be produced by combined, but not separate, administration of a selective D1 agonist (SKF 38393) and a selective D2 agonist (quinpirole) (Moore and Axton, 1988). Therefore, as in rats, concurrent activation of the D1 and D2 receptors in mice seems to be required to activate motor behavior, indicative of D1/D2 synergism.

Synergism is a well-known concept in the field of pharmacology, whereby the effect of two agonists given concomitantly is greater than the sum of their effects when given separately. In the case of DA receptor interaction, the synergism is extreme because separate stimulation of each receptor sub-type elicits no response of its own. The absence of a response is most evident

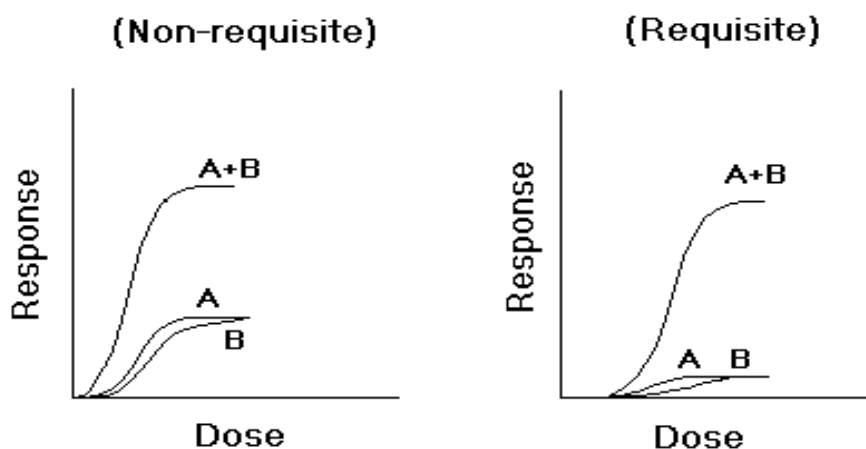Figure 2. Dose-response curves representing the non-requisite and requisite forms of synergism. Drugs A and B are given separately and concomitantly in each graph, with their dose-response curves superimposed.

when immediate-early gene expression is examined (LaHoste et al., 1993). Thus, the term "requisite synergism" has been introduced to distinguish this phenomenon from the traditional "non-requisiste" synergism (Fig. 2).

Breakdown in D1/D2 synergism

The D1/D2 receptor interactions described above hold true under normal conditions, but the requisite D1/D2 synergism is broken down with prolonged DA depletion in the striatum. When reserpine, a catecholamine depleting agent, is repeatedly administered systemically to a normal rat, selective D1 or D2 agonists become sufficient to independently elicit heightened locomotion and stereotypic behaviors (Arnt, 1985; LaHoste and Marshall, 1993) and increased inhibition of cells in the caudate/putamen (Hu et al., 1990). In mice as well, repeated reserpine treatment allows selective D1 or D2 agonists to induce motor activity (Pichler and Pifl, 1989; Ferre et al., 1994). Another method of D1/D2 breakdown is the administration of the selective dopaminergic toxin 6-hydroxydopamine (6-OHDA) to the nigrostriatal pathway. The resulting death of the dopaminergic neurons and depletion of endogenous DA liberates the respective DA receptors from their normal interdependence. Following a unilateral 6-OHDA lesion, independent stimulation of D1 or D2 receptors will induce rotation (Arnt and Hyttel, 1984), which is indicative of an asymmetric breakdown in D1/D2 synergism. In rats with bilateral 6-OHDA lesions, the animals display heightened locomotor activity and motor stereotypy in response to D1 or D2 agonists alone (LaHoste and Marshall, 1992). In addition to their effects on D1/D2 synergism, reserpine or 6-OHDA treatments result in a pronounced supersensitivity of the receptors to agonist. Thus, there is an invariable association between the breakdown in D1/D2 synergism and DA receptor supersensitivity (Hu et al., 1990; LaHoste and Marshall, 1992).

D1/D2 receptor co-localization

There are several theories which attempt to explain the mechanism behind D1/D2 synergism and the supersensitivity related to its breakdown. One theory relies on co-localization

of D1 and D2 receptors, which would imply that an intracellular cascade of events would be responsible for the change in sensitivity and breakdown in synergism (Fig 3A). A different hypothesis, based on the assumptions of separate populations of striatal neurons containing D1 vs. D2 receptors, would require some intercellular form of communication to take place. Since medium spiny striatal neurons have dense axon collaterals that synapse nearby the soma, collateral inhibition might allow for this interneuronal communication (Fig. 3B). Due to differing results, the cellular localization of the respective DA receptor types has been controversial.
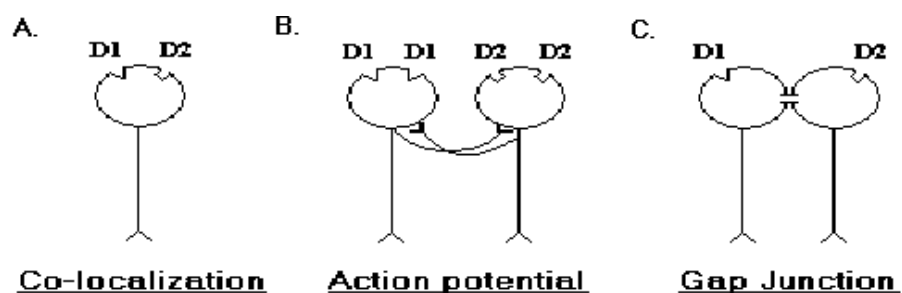
Figure 3. (A) A neuron with both D1 and D2 type receptors is depicted. (B) Two neurons expressing distinct subfamilies of DA receptors communicates via action potentials. (C) Two neurons with distinct subfamilies of DA receptors communicate via gap junctions.

Most *in situ* hybridization studies have shown that distinct sub-populations of striatal projection neurons express mRNA for mainly D_1 receptors, while a different sub-population of striatal projection neurons express mRNA for mainly D_2 receptors. Additionally, these two sub-populations differ with respect to their anatomical targets and to the peptide(s) that are co-localized in these uniformly GABA-ergic neurons (Fig. 4). The D_1–expressing neurons project primarily to the SNr (i.e., they are striatonigral and constitute the "direct" pathway depicted in Fig. 1) and co-express the peptides substance P and dynorphin, as well as GABA. The D_2–expressing neurons project mainly to the GP (i.e., they are striatopallidal and constitute the

Caudate-Putamen
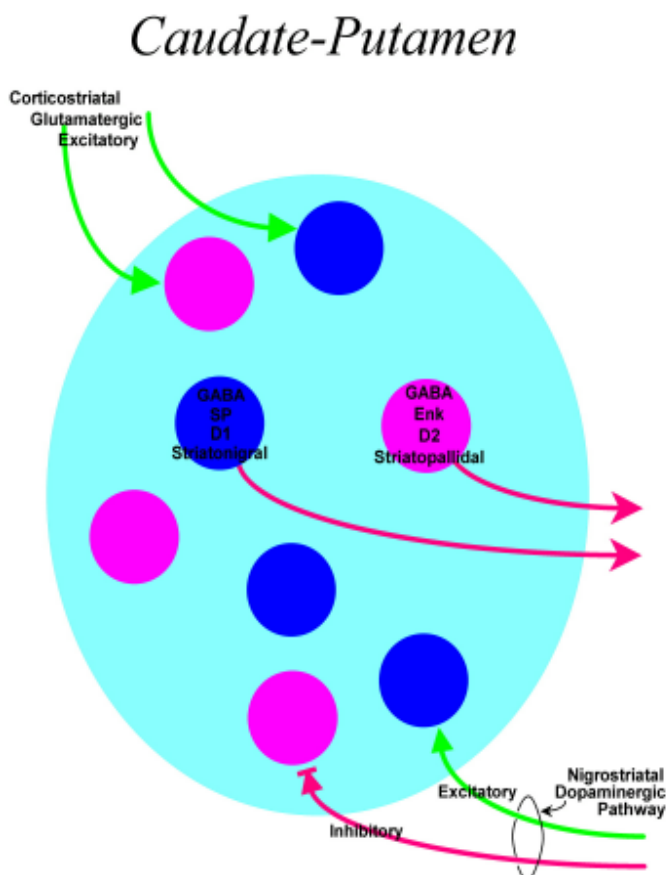
Figure 4. Schematic representation of the CPu. Only medium spiny neurons (which comprise 90-95% of striatal cell bodies) are shown. The two major inputs to the CPu are the corticostriatal glutamatergic inputs and the dopaminergic nigrostriatal inputs. D1 and D2 receptors are located on separate populations (with equal numbers) of medium spiny neurons that differ with respect to their anatomical projections and the peptide(s) that they co-express.

first step in the "indirect" pathway) and co-express enkephalin with GABA (Gerfen, 1992; Le Moine and Bloch, 1995). Immunocytochemistry at the electron microscope level using antibodies for D_1 and D_2 receptors also revealed distinct sub-populations of medium spiny neurons in striatum (Hersch et al., 1995). Furthermore, receptor binding studies have shown preferential binding of D_1 receptors in the SNr, where striatonigral neurons synapse, while the D_2 receptor binding alone is found in the GP where striatopallidal axons terminate (Harrison et al., 1990; Richfield et al, 1989).

In seeming contrast to the D1/D2 co-localization evidence presented above, studies in which mRNA from single cells was amplified billions of times by multiple rounds of polymerase chain reaction (PCR) reported a sizable population of striatal neurons that co-express D_1 and D_2 mRNA (Surmeier, 1992). However, subsequent papers from these investigators suggest that while D_1 receptors may be co-expressed with D_3 or D_4 receptors, they are rarely co-expressed with D_2 receptors *per se* (Surmeier, 1996). Similarly, D_2 receptors are co-localized with D_5 receptors, but not D_1 receptors *per se*. When the initial experiments of Surmeier et al. (1992) used standard (single round) PCR (as opposed to multiple rounds), little co-localization of D_1 and D_2 receptors was seen. This finding is consistent with their subsequent work (Surmeier, 1996) and with the *in situ* hybridization and EM-level immunohistochemistry studies (Hersch et al., 1995; LeMoine and Bloch, 1995). Taken together, these results strongly support the hypothesis that D_1 and D_2 receptors are located on separate populations of striatal projection neurons, as indicated in Fig. 3B and 3C.

Nonetheless, Surmeier's studies raise the possibility that behavioral synergism could be due to D_1/D_3, D_1/D_4, or D_2/D_5 interactions within a given neuron since all previous studies relied on pharmacological agents that do not distinguish between sub-types of a receptor family. To test this possibility directly, LaHoste et al. (2000) employed newly developed antagonists that are selective for D_2, D_3, and D_4 receptors. Interestingly, the use of selective D_2, D_3, and D_4 antagonists revealed that only D_2 receptor blockage significantly reduced Fos immunoreactivity in the striatum (a reliable marker for combined D1/D2 activity in normal rats) (LaHoste et al., 2000). So, assuming the two main types of DA receptors (D_1 and D_2) are located on separate neurons and project mainly to separate areas, the mechanism allowing the neurons to interact, to bring about requisite synergism and supersensitivity, must be interneuronal.

Interneuronal communication

The most common form of interneuronal communication occurs at the synapse via action potentials. Alterations in the number and frequency of action potentials are often a means of modulation for many neuronal circuits. If D_1 and D_2 receptors reside on separate neurons that communicate via action potentials, thereby giving rise to D_1/D_2 synergism, then blockade of action potentials should prevent D_1/D_2 synergism. To test this hypothesis, LaHoste et al. (2000) administered the fast sodium channel blocker tetrodotoxin (TTX) intrastriatally to rats, and found that D_1/D_2 synergism persisted (as evidenced by Fos expression) despite blockade of action potentials. Moreover, the observed *c-fos* activity in the absence of action potentials cannot be explained by an interaction between co-localized D_1 & D_3, D_1 & D_4, or D_2 & D_5 receptors, because the administration of selective D_3 or D_4 antagonists did not prevent Fos expression in amphetamine-treated rats, whereas a D_2 selective antagonist did (LaHoste et al., 2000).

Electrotonic Coupling via Gap Junctions

An alternative explanation for the body of evidence described above could be that the D_1 and D_2 receptors are interacting by means of gap junctions. Gap junctions are made of two hemi-channels or connexons that link the cytoplasm of two cells. Each cell produces its own hemi-channel consisting of a hexamer of connexin proteins. Although each connexon can be in an open or closed state independently, when the two connexons are open, they allow electrical current and some small molecules to passively travel from cell to cell (Feldman et al., 1997). Electrotonic coupling via gap junctions in the CPu and nucleus accumbens have been shown to exist and to be modulated by different DA agonists and antagonists, as revealed by dye-coupling (O'Donnell and Grace, 1993; Onn and Grace, 1994). The percentage of striatal neuron dye-coupling increased dramatically in rats receiving apomorphine (from 17% to 82% of injected

cells) (Onn and Grace, 1994). Both electrolytic and neurochemical DA-depleting lesions in the striatum, treatments that would be expected to result in a breakdown in D1/D2 synergism and agonist supersensitivity, resulted in an increase in dye coupling between striatal neurons when compared to control animals (Cepeda et al., 1989). Furthermore, gap junctions in the retina have been known to be modulated by DA, which seems to be the mechanism for light and dark adaptation (Godley and Wurtman, 1988; Hampson et al., 1992; He et al., 2000). So, gap junctions, specifically their constituent parts called connexins, have garnered newfound attention in the field of neuroscience.

The identification of different connexin gene sequences has allowed researchers to localize their primary sites of expression using various *in vitro* techniques. In particular, connexin 32 (Cx32) and connexin 36 (Cx36) have been cataloged in rat and mouse striatum (Matsumoto et al., 1991; Condorelli et al., 2000). Cx32 is most prevalent in the liver, but it is also found in the retina, primarily in oligodendrocytes (Dermietzel et al., 1989). Cx36 is found exclusively in neurons in the brain, particularly in the inferior olive, retina, and olfactory bulbs, but also in striatum (Condorelli et al., 2000; Rash et al., 2001). Recently, it has been discovered that Cx36 is essential for the propagation of rod-mediated signals in the retina of mice (Deans et al., 2002). These two connexins are prime targets for research involving plasticity in the striatum concerning DA activity. So, DA-depletion in the striatum leads to: independence of D1/D2 receptors to activate motor stereotypy; independence of D1/D2 receptors to allow D1 agonists to induce *c-fos* expression; and increases in dye-coupling and connectivity in the striatum.

Hypothesis and Rationale

The evidence described above suggests that connexins may play a role in D1/D2 synergism in the striatum. These connexins (Cx32,Cx36) are abundant in neural tissue.

Depletion of DA in striatal tissue, which would normally bring about a breakdown in D1/D2 synergism in a rodent, leads to increases in gap junction dye coupling. Therefore, mice which have been genetically engineered so as not to express Cx32 or Cx36 proteins should behave differently than their wild-type littermates on a behavioral test, after separate or concomitant administration of selective D1 and D2 agonists. Climbing behavior in mice has shown to be a consistent indicator of DA activity, and is an easily observable and quantifiable form of behavior. I hypothesize that genotype differences (i.e., -/- or +/+) between littermate mice of either Cx32-engineered or Cx36-engineered strains, will show behavioral differences with respect to stereotyped motor behavior (climbing) elicited by separate or combined D1 and D2 receptor stimulation.

Methods

Animals

Using homologous recombination, Paul & Willicke and their colleagues generated founder mice in which either the Cx32 or Cx36 gene was "knocked out" (Nelles et al., 1996; Deans et al., 2001). As is standard procedure, embryonic stem cells from mice of strain "129" were implanted into blastocysts of pregnant female mice of the strain C57BL/6. The Cx32 and Cx36 transgenic mice used in these experiments were bred at the University of New Orleans, in the Department of Psychology rodent colony. The original male and female breeders used to start the colony were generously donated by Dr. David L. Paul (Harvard University). The Cx36 gene is located on autosome 2, thus breeding of heterozygous males and females yields a complement of two homozygous genotypes (both wildtype (WT) and knockout (KO)) and a heterozygote (Het) genotype. Due to the location of the Cx32 gene on the murine X chromosome, males only possess one copy of the gene. Therefore, males can be either wildtype or *hemi*zygous for the Cx32 knockout allele, but the hemizygotes are equivalent to the autosomal homozygotes for the knockout allele since no Cx32 protein is made.

Because of the diversity of genotypes present in every litter of offspring, each mouse pup must be subjected to "DNA fingerprinting" for the gene of interest. After weaning, each mouse was genotyped using genomic DNA purified from tail biopsies taken under anesthesia. Tail biopsies were digested overnight at 55°C in proteinase K. After centrifugation, an equal volume of isopropanol was added to the supernatant to precipitate high molecular weight genomic DNA. Genomic DNA for each mouse was amplified by PCR using the following protocol: 1) for each

connexin gene (i.e., Cx32 or Cx36), a cocktail was made containing: 10X buffer, 25 mM Mg^{++}, 2.5 mM dNTP's, a 3' primer that is common to both WT and KO alleles, a 5' WT-specific primer, and a 5' KO-specific primer at prescribed concentrations; 2) the cocktail was distributed equally into individual tubes; 3) 0.5-1 μg of the genomic DNA for each mouse was added to a single tube, along with Taq polymerase; 4) the tubes were then placed in a thermocycler (set for 30 cycles) to amplify the DNA. After amplification, the DNA was analyzed using gel electrophoresis in a 1% agarose gel. Genotype was determined by the presence of a WT and/or KO band in each lane of the gel (Fig. 5). Adult male Cx32–deficient (Cx32 KO) mice (n=12)
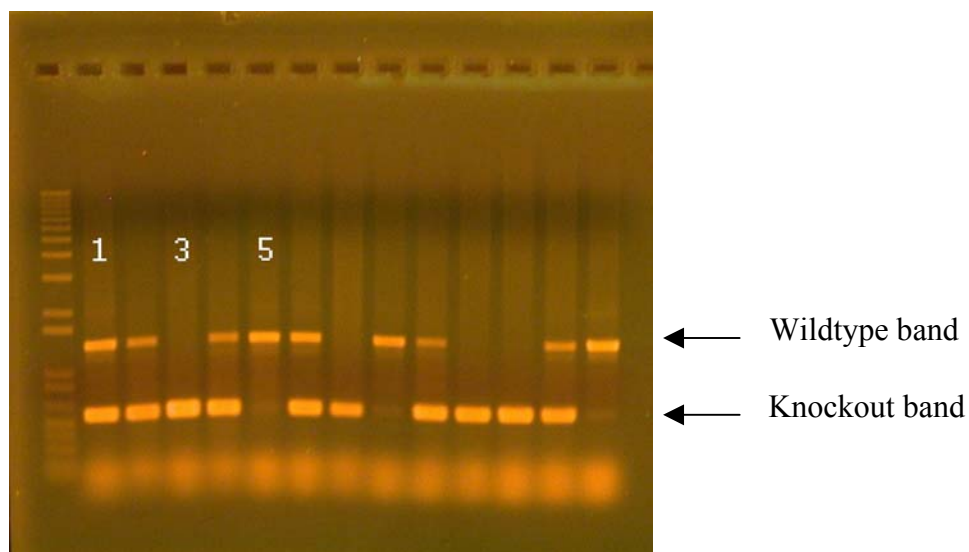
Figure 5. An agarose gel displaying the different bands of DNA amplified by PCR for the Cx36 mice sampled. Lanes 1, 3, and 5 are examples of the three possible genotypes: Lane (1) shows a heterozygous mouse, which possesses one KO allele and one WT allele; Lane (3) shows a KO mouse, which is homozygous for the transgenic Cx36 gene; Lane (5) shows a WT mouse, which is homozygous for the normal Cx36 gene.

and their wildtype (Cx32 WT) littermates (n=12), along with adult male and female Cx36-deficient (Cx36 KO) mice (n=12) and their wildtype (Cx36 WT) littermates (n=12) were used in this experiment. The mice weighed between 20-30g at the beginning of the study, and were between 2- to 5-months old. They were group housed at 3 – 10 mice per same-sex cage, and they were kept on a 12-hour light/dark cycle from 7:00 am – 7:00 pm, with free access to food and water. All the mice were maintained and used in accordance with the guidelines for animal

care and experimentation established by the National Institutes of Health and the University of New Orleans Institutional Animal Care and Use Committee (approved protocol #024).

Apparatus

There were six separate cylinders (36 cm high, 18 cm diameter, 1 cm mesh size) in which the mice were tested simultaneously. The cylinders were composed of a plastic mesh material. The tops of the cylinders were covered by cardboard, with bedding material spread along the bottom. While in the cylinders, the animals' climbing behavior was recorded by a video camera, and later viewed for quantification by two observers blind to the conditions. The experiment was set up in an isolated room to minimize outside stimuli that might influence the animals.

Drug treatments

Climbing behavior was observed following each of four DA receptor stimulation treatments: A) control, B) D1 receptor stimulation, C) D2 receptor stimulation, D) D1/D2 receptor stimulation. All mice were tested under all agonist conditions using a Latin square design. Each treatment/test was separated by one week. As seen in Figure 6, the heterotypic antagonist was used to ensure agonist stimulation of only the intended DA receptor subfamily. This was necessary in order to block any endogenous DA from binding with the D1 or D2 receptors. Agonist injections were given fifteen minutes after antagonists, at which time the DA antagonists had reached blood concentrations capable of blocking >99% of their specific receptor. For agonist stimulation of D1 receptors alone, the D2 antagonist eticlopride (0.1 mg/kg, i.p.) and the D1 agonist SKF 38393 (30 mg/kg, i.p.) were administered. For agonist stimulation of D2 receptors alone, the D1 antagonist SCH 23390 (0.1mg/kg, s.c.) and the D2 agonist quinpirole (1.6 mg/kg, i.p.) were administered. The D1/D2 stimulation group received an initial injection of saline (10 mg/kg), followed by combined administration of SKF 38393 (30

mg/kg, i.p.) and quinpirole (1.6 mg/kg, i.p.). The control group received two saline (10 mg/kg)

injections at comparable times to the other groups. All drug injections were administered in a

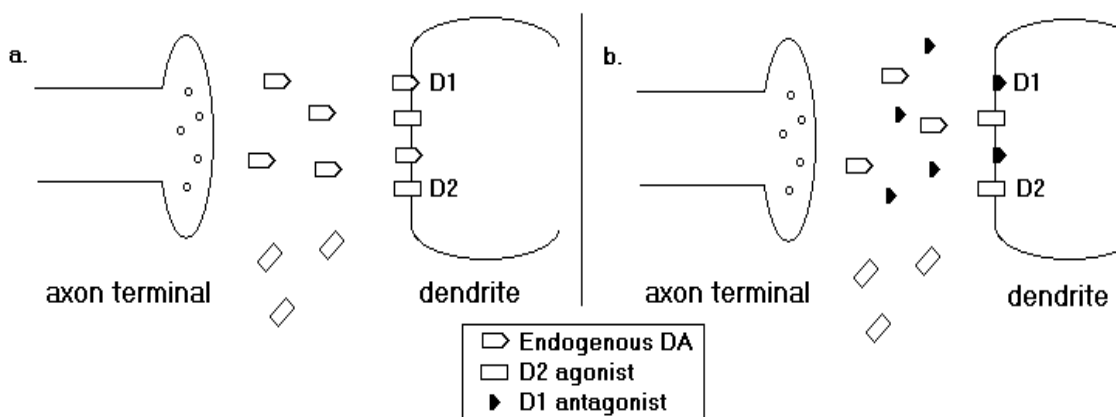volume of 10 ml/kg of body weight.

Figure 6 (a.) The D2 agonist enters the synapse in the presence of endogenous DA. The postsynaptic neuron
experiences D1 and D2 receptor activation. (b.) The D1 antagonist is administered prior to the D2 agonist, thereby
preventing endogenous DA from activating the D1 receptors.

Procedure

The mice remained in the cylinders for a total of ninety minutes, with a climbing

behavior score being taken every five minutes. Their scores were based on the number of paws

the mice had on the mesh cylinder at a given moment. The scores could range from 0 (no paws

on cylinder) to 4 (all paws on cylinder) (Protais et al., 1976).

First, each mouse was placed in a cylinder for fifteen minutes to establish a baseline of

climbing behavior. Then, the mice received their first injection (antagonist or saline), followed

by another fifteen-minute period of observation. Finally, the second injection (agonist(s) or

saline) was administered, and the mice remained in the cylinder for the remaining sixty minutes

of the session (See Table 1). The experimenter was only present in the room at the time of

injection to minimize variability in the animals' behavior.

Table 1.

	Time Course for each Session		
	0 – 15 min.	**15 – 30 min.**	**30 – 90 min.**
DA Receptor(s) stimulated	**Placed in cylinder**	**First Injection**	**Second Injection**
None	--	Saline	Saline
D1 only	--	Eticlopride	SKF 38393
D2 only	--	SCH 23390	Quinpirole
D1 + D2	--	Saline	SKF + Quin.

Data Analysis

Data for each strain of mouse was analyzed separately. Climbing scores were subjected to a mixed-design three-way analysis of variance (ANOVA) with one between-subjects factor (genotype) and two within-subjects factors (drug treatment and time). Significant main effects were further analyzed by Newman-Keals *post-hoc* tests; significant interactions were analyzed by performing tests of simple effects. All statistical analyses were performed on SPSS for Windows (Version 11.0).

Results

Connexin32 mice

Time post-agonist was not a significant factor in any analysis; therefore, climbing scores were averaged across time points for all subsequent analyses. Regardless of genotype, Cx32 mice showed stereotyped climbing behavior only following combined D1/D2 stimulation (Figure 7). A 2 x 4 (Genotype x Drug Treatment), mixed, repeated measures
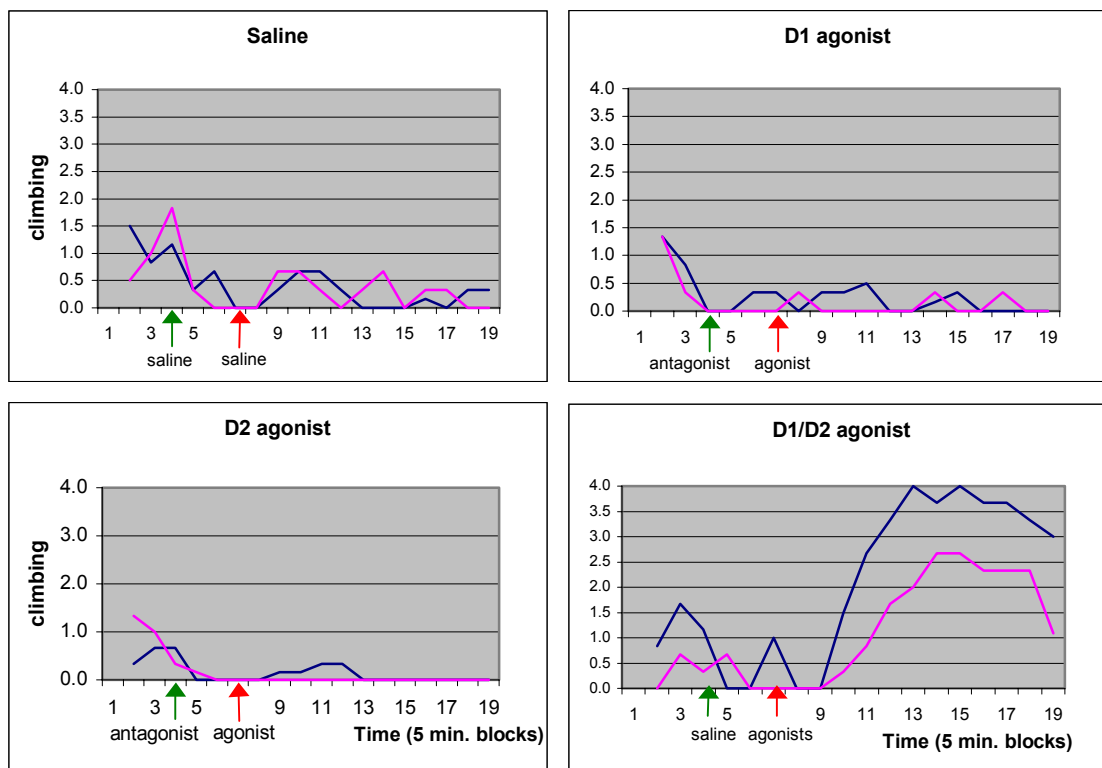

Figure 7. Climbing scores across time under all treatments for Cx32 WT(blue) and KO(pink) mice. The arrows indicate the times of the first(green) and second(red) injections.

ANOVA revealed a significant main effect for Drug Treatment, $F_{(3,66)} = 74.51$, $p < 0.0001$, a significant main effect for Genotype, $F_{(1,22)} = 5.46$, $p < 0.025$, and a significant Drug x

Genotype interaction, $F(3,66) = 6.66$, $p < 0.01$. *Post hoc* Newman-Keuls tests on the Drug

Treatment effect showed that the combined agonist group differed significantly from all other

agonist or saline treatments, none of which differed significantly from each other ($p < 0.05$).

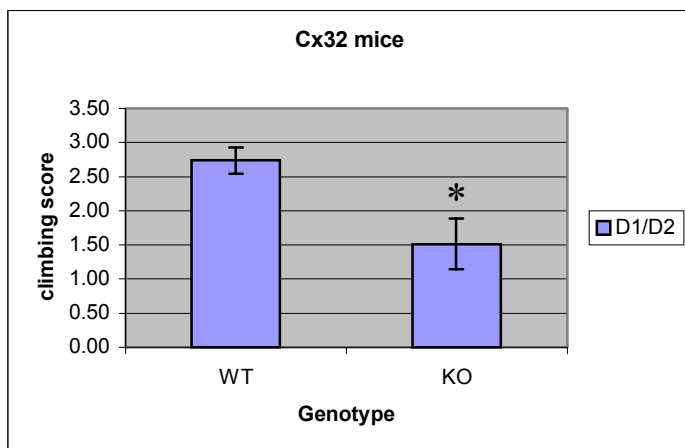Figure 8. The average climbing score collapsed over time post-agonist in the D1/D2 treatment group for the Cx32 WT and KO mice. (* signifies $p < .01$)

Tests of Simple Effects showed that the significant interaction results from the fact that Cx32

KO mice displayed lower climbing scores compared to their WT littermates when given

combined D1/D2 stimulation but not following any of the other drug treatments (Figure 8).

Connexin36 mice

Identical analyses to those performed on time-averaged climbing scores for Cx32 mice

showed that, regardless of genotype, Cx36 mice showed sustained stereotyped climbing behavior

only following combined D1/D2 stimulation (Figure 9). A 2 x 4 (Genotype x Drug Treatment),

mixed, repeated measures ANOVA revealed a significant main effect for Drug Treatment,

$F(3,66) = 3.88$, $p < .05$, but no significant effect for Genotype, $F(1,22) = 1.88$, $p = .184$, n.s., or

for a Drug Treatment x Genotype interaction. As in Cx32 mice, *post hoc* Newman-Keuls tests

on the Drug Treatment effect showed that the combined agonist group differed significantly from

all other agonist or saline treatments, none of which differed significantly from each other ($p <$

0.05).
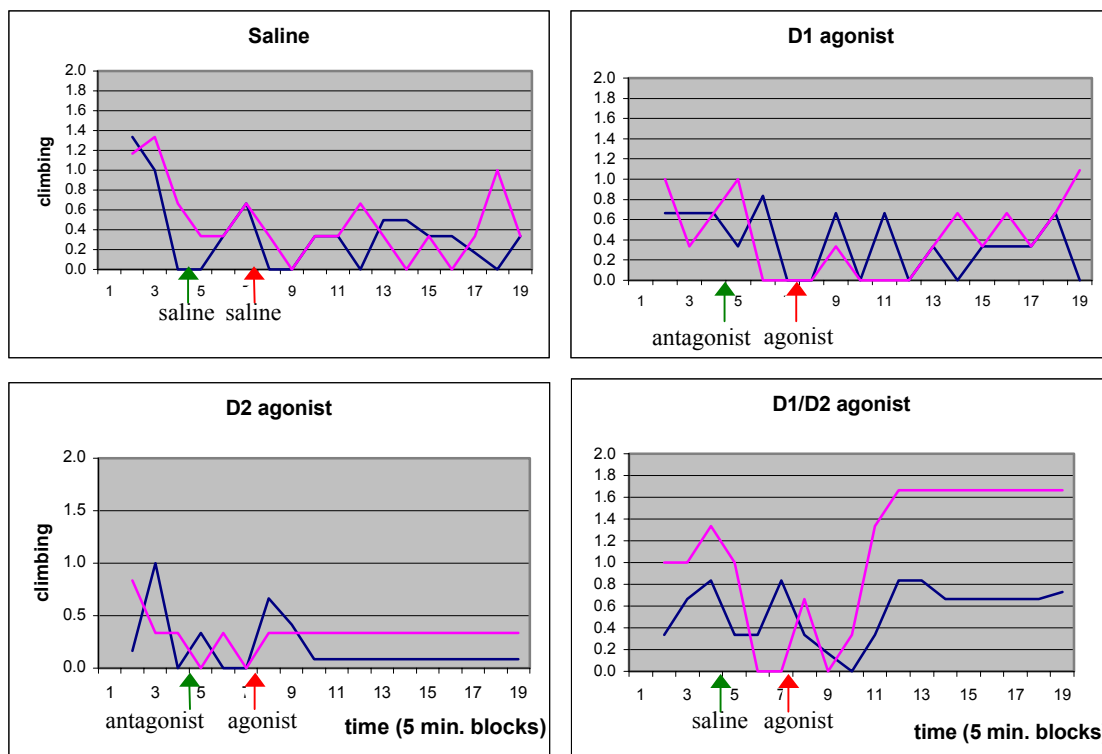
Figure 9. Climbing scores across time under all treatments for Cx36 WT(blue) and KO(pink) mice.

Strict adherence to the rules of inferential statistics requires that nonsignificant main effects or interactions not be analyzed further. However, there was such a high degree of variability in the scores of Cx36 mice following combined D1/D2 administration that further investigation into this variability was determined to be of potentially important heuristic value. Although Genotype was not a significant factor, Cx36 KO mice displayed higher average climbing scores than did their WT littermates following combined D1/D2 stimulation (mean KO = 1.31; mean WT = 0.57) (Figure 10). This trend is in the opposite direction to that observed in Cx32 mice. Furthermore, almost all of this trend can be accounted for by higher climbing scores in female KO mice relative to their WT littermates or to males of either genotype, $F(1,20) = 2.35$, $p = 0.14$ (Figure 11).
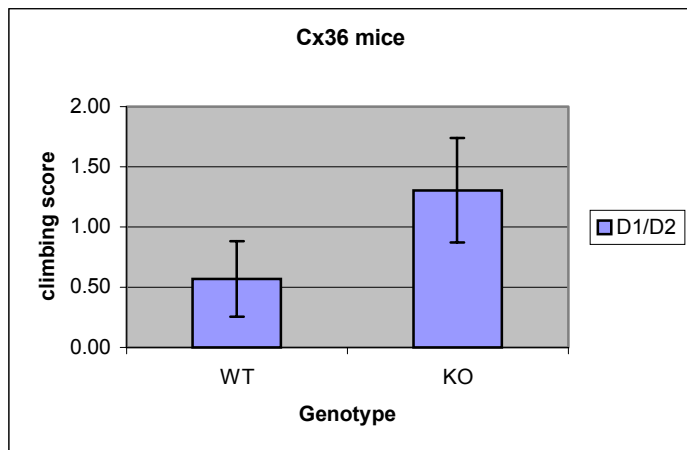
Figure 10. The average climbing score collapsed over time post-agonist in the D1/D2 treatment group for the Cx36 WT and KO mice. The difference between the means is not statistically different.

The observation described above draws attention to an additional anomaly in the data from Cx36 mice. Re-examination of Figure 9 suggests that, although there was a significant main effect for Drug Treatment (but no significant interaction of this variable with Genotype), the *control* WT mice may not be showing significant climbing behavior even in response to combined D1/D2 agonists. In fact, when WT mice are analyzed separately, there is no significant effect of Drug Treatment. The same holds true for KO male mice. These anomalies are addressed in the Discussion.

Figure 11. Bar graph displaying the average scores (post-agonist) for male and female Cx36 WT and KO mice during the combined D1/D2 agonist treatment.

Discussion

Despite its restricted anatomical distribution within mammalian brains, DA has a widespread influence on movement, motivation, attention, reinforcement and cognition. Therefore, disturbances in DA function play a major role in many behavioral disorders including Parkinson's disease, depression, drug abuse, schizophrenia and ADHD. A hallmark of normal dopaminergic function is the requisite synergistic interaction 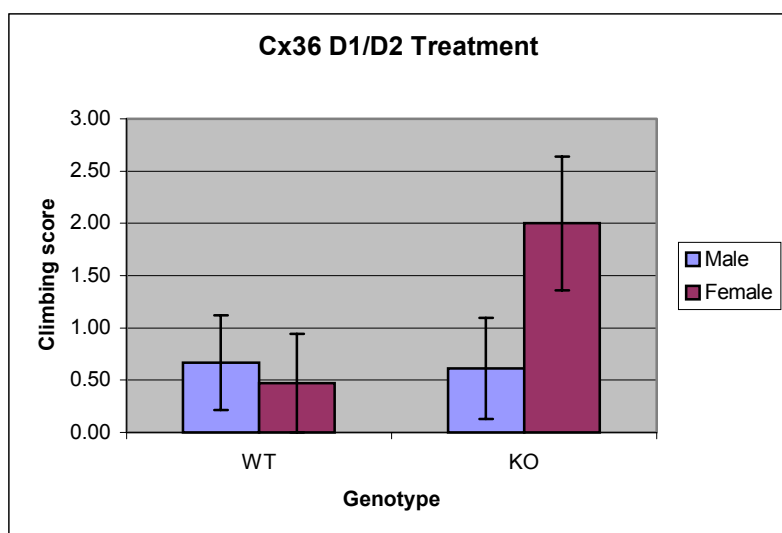between D1 and D2 subtypes of DA receptors. Previous work indicates that this synergism cannot be accounted for either by receptor co-localization within the same neuron or by classical interneuronal communication via action potentials. These findings led to the main hypothesis of the present thesis which is that electrotonic coupling between neurons via gap junctions may play a role in D1/D2 synergism. Since gap junctions are composed of channels formed by specific proteins called connexins, we tested this hypothesis by examining D1/D2 synergism in mice with targeted disruptions of either of two connexin genes, namely Cx32 and Cx36.

Of all the currently known connexins, Cx32 and Cx36 were prime candidates for research involving the dopaminergic system due to their prevalence in nervous tissue and their presence in the striatum. The main finding of the present thesis research is that there is not an absolute requirement for Cx32 protein in the mouse brain for the manifestation of D1/D2 synergism; the role of Cx36 is less clear. Regardless of genotype, Cx32 mice displayed stereotypic climbing behavior only following combined agonist stimulation of D1 and D2 DA receptors. Previous research has shown that combined administration of D1 and D2 agonists is required in normal

mice to elicit cage-climbing behavior (Moore and Axton, 1988). The lack of Cx32 in these transgenic mice does not appear to change the requirement of concomitant D1 and D2 receptor stimulation, as D1 or D2 agonists given alone do not result in significantly different scores among the genotypes of either strain. Nonetheless, there were genotype differences in the magnitude of the response to combined D1/D2 stimulation.

Although the absence of Cx32 does not appear to eliminate requisite D1/D2 synergism, the behavioral response to combined D1/D2 agonist treatment was significantly diminished in mice lacking this protein compared with their WT littermates. Thus, Cx32-constituted gap junctions may have a modulatory influence on this phenomenon. The significant variation in climbing response between the KO and WT mice is likely related to the lack of Cx32, which could be directly affecting the synchronous firing of GABA-ergic interneurons in the striatum. A recent study by Sutor and colleagues (2000) found that Cx32 null mutants displayed a dysfunction of inhibitory synaptic transmission in neocortex, which was associated with decreased myelination (Sutor et al., 2000). The neocortex provides the major input to the striatum (CPu) where excitatory corticostriatal synapses oppose DAergic inputs on the same dendritic spines of striatal output neurons; this could explain the decreased behavioral response to dopaminergic stimulation. A similar effect at the level of the medium spiny striatal output neurons could also explain diminished behavioral response to DA agonists in Cx32 KO mice. As shown in Figure 1, DA-mediated behavior is affected by inhibition of SNr neurons. DA, by facilitating the direct inhibitory striatonigral pathway and inhibiting the indirect striatofugal pathway inhibits SNr neurons, the main output of the basal ganglia. If there were a deficiency of GABA-ergic inhibitory transmission emanating from the striatum, a diminished behavioral response to D1/D2 agonists would be expected.

With regard to Cx36 mice, a strict interpretation of the statistical analyses leads to the conclusion that these mice, like Cx32 mice, displayed intact D1/D2 synergism; however, unlike Cx32 mice, the behavioral response of these mice to combined D1/D2 stimulation did not differ between WT and KO mice. Although there was no statistically significant difference between Cx36 KO and WT mice, there was a trend ($p = 0.18$). Interestingly, that trend was opposite to the effect seen in Cx32 mice: Cx36 KO mice tended to show higher stereotypy scores in response to combined D1/D2 stimulation relative to their WT littermates. The non-significance of this difference may be partially attributable to increased variability of behavioral scores within this strain, which, in turn, may be due to the fact that it was necessary to include both males and females in this study (Cx32 animals were all males). Thus, there may be a genotype-by-gender effect. An analysis of this potential effect requires reducing the sample sizes in each cell by half, thereby reducing the power of the statistical test. Nonetheless, a nonsignificant trend ($p = 0.14$) for such an interaction was obtained. Cx36 KO females showed climbing scores (to combined D1/D2 stimulation) that were nearly quadrupled relative to WT females or to males of either genotype (see Figure 9). Indeed, most of the difference between Cx36 KO and WT mice, in general, can be accounted for by the increased scores in female KO mice. Thus, Cx36-constituted gap junctions may play a modulatory role in D1/D2 synergism, but if so, this effect would be restricted to female animals.

In light of the trend described above, a further source of variation in the data from Cx36 mice is the state of estrus of the female mice, which was not controlled for in the present experiment. A number of studies have shown that the level of DA-stimulated behavior, such as rotations and stereotyped behaviors, can vary depending on the animal's current stage of the estrous cycle (Joyce and Hartesveldt, 1984; Becker and Cha, 1989; Diaz-Veliz et al., 1994).

However, variability due to the estrous cycle alone cannot fully explain this disparity, since the WT females did not exhibit a similar effect. The fluctuating reproductive hormones of the estrous cycle and the absence of Cx36 could be interacting to produce this effect. Accounting for the estrous cycle in later experiments using daily vaginal lavages would allow greater consistency in behavioral testing, and an additional way to measure the interaction between the female reproductive hormones and genotype.

The observations described above draw attention to an additional anomaly in the data from Cx36 mice. Re-examination of the data shows that the *control* WT mice may not be showing significant climbing behavior even in response to combined D1/D2 agonists. In fact, when WT mice are analyzed separately, there is no significant effect of Drug Treatment. The same holds true for KO male mice. Does this mean that these animals do not have intact D1/D2 synergism? For several reasons, that conclusion cannot be drawn from the present data. First, the absence of D1/D2 synergism is always manifested by a significant functional response to separate agonist stimulation of D1 or D2 receptors alone, an effect that is not seen in the present study. Second, the ability of a D1 or D2 antagonist alone to induce catalepsy (lack of motor movement, muscle rigidity, splayed hind legs and "Straub" tail effect in rodents) is evidence of D1/D2 synergism since motor behavior is mediated by endogenous DA interacting with its D1 and D2 receptors. In the present study, all mice given either a D1 or a D2 antagonist invariably showed clear and obvious signs of catalepsy. Thus, D1/D2 synergism was operative in all of the mice used in the present study, regardless of strain or genotype.

A more likely explanation for the failure of Cx36 mice (except for female KO's) to display significant climbing following combined D1/D2 agonist treatment is that these mice are subsensitive to DA agonists and that significant climbing behavior might have been observed

had higher doses been used. (The doses used in the present study were chosen on the basis of pilot studies on control and Cx32 mice.) This subsensitivity could be due to the residual presence of a significant number of 129-strain genes (see Methods), as mice of this strain are known to be subsensitive to DA agonists (Schlussman et al., 1998). [Due to differing degrees of back-crossing of Cx32 vs. Cx36 mice with control C57BL/6 mice—which after many generations ultimately eliminates most 129 genes—it is not unlikely that such an effect would be present in one strain and not the other.] Other factors that were difficult to control for in this experiment include possible developmental compensation for the lack of Cx32 or Cx36 in the KO mice. As in all experiments using genetically engineered animals, the target gene is inactivated beginning at the single cell stage. As the animal develops, absence of a given protein may elicit a compensatory developmental process, thereby minimizing or eliminating potential deficits. In the present experiments, some degree of compensation for the lack of these gap junction proteins could have occurred developmentally and obscured the natural role of the protein. This is a problem inherent in all knockout experiments; that is, the effect of knocking out a protein all throughout development does not result in the same phenotype as acutely blocking the effect of a protein in adulthood.

The present research hypothesizes that gap junctions modulate DAergic activity. Connexins 32 and 36 were chosen for study based on previous research indicating that they were the most likely known connexins to be involved. However, gap junctions made up of other as yet undiscovered connexins may be the crucial ones in this regard. The connexin family is large and new connexins are being discovered on a regular basis.

A potential solution to both of the problems described above would be to acutely manipulate the "open" or "closed" state of gap junctions. However, no currently known

pharmacological agents can selectively block gap junctions without exerting simultaneous effects on other tissue that are unrelated to electrotonic coupling.

In summary, the present experiment found D1/D2 requisite synergism to be intact in mice with targeted deletions of Cx32 or Cx36 genes. The degree of behavioral response to D1/D2 stimulation was diminished in the Cx32 KO mice when compared to their WT littermates. Cx36 KO mice displayed a trend toward higher climbing scores relative to Cx36 WT mice. This trend is not significant, perhaps due to increased variability introduced by the inclusion of female mice whose estrous cycle could be interacting with the absence of Cx36 to produce elevated climbing scores.

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