Title: Intrinsic regulators of the action potential waveform control dopamine release to shape behavior

One sentence summary: We show that ion channels in midbrain dopamine neurons are critical for patterning neurotransmitter release to regulate reinforcement learning.

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Abstract:

Despite the widely known role of dopamine in reinforcement learning, how the patterns of dopamine release that are critical to the acquisition, performance, and extinction of conditioned responses are generated is poorly resolved. Here, we demonstrate that the coordinated actions of two ion channels, Kv4.3 and BKCa1.1, control the pattern of dopamine release on different time scales to regulate separate phases of reinforced behavior in mice. Inactivation of Kv4.3 in VTA dopamine neurons increases pacemaker activity and excitability that is associated with increased ramping prior to lever press in a learned instrumental response paradigm. Loss of Kv4.3 enhanced performance of the learned response and facilitated extinction. In contrast, loss of BKCa1.1 increased burst firing and phasic dopamine release that enhanced learning of an instrumental response. Inactivation of BKCa1.1 increased the reward prediction error that was associated with an enhanced extinction burst in early extinction training. These data demonstrate that temporally distinct patterns of dopamine release are regulated by the intrinsic properties of the cell to shape behavior.

36 **Introduction**

37 Reinforcement learning is an essential process by which an organism links environmental
38 stimuli and actions with outcomes. Dopamine-producing neurons in the ventral tegmental area
39 (VTA) of the midbrain facilitate reinforcement learning to promote the performance of goal-
40 oriented behavior through modulation of information processing in the forebrain, most notably in
41 the nucleus accumbens (NAc) (*1*). Distinct patterns of dopamine release that occur on different
42 time scales during the acquisition, performance, and extinction of a conditioned response are
43 thought to underlie dopamine function within these contexts (*2-5*). Despite the evidence that
44 dopamine is critical for reinforcement learning (*1-3*), how the intrinsic properties of the cell
45 contribute to dopamine release, and whether patterns of release observed during learning and
46 subsequent performance of a conditioned response influence patterns of release during extinction
47 is not well resolved. Moreover, recent findings suggest that dopamine release dynamics can occur
48 independently of the activity patterns at the cell body (*5*) raising the possibility that the firing
49 properties of dopamine neurons may be dispensable for some aspects of reinforcement learning.

50 VTA dopamine neurons display spontaneous action potential firing in the absence of
51 afferent inputs (*6*) that is regulated by a suite of voltage-gated ion channels (*7-9*). Potassium
52 currents shape the action potential waveform to regulate the frequency and pattern of firing (*7-10*).
53 Kv4.3 subunits contribute to A-type potassium currents (I_A) in dopamine neurons (*8*) that facilitate
54 repolarization and control the frequency of firing (*8*). BKCa1.1 subunits are the essential subunit
55 of voltage-sensitive, calcium-activated big conductance potassium. (BK) channels conduct BK
56 currents (I_{BK}) that mediate the initial phase of the action potential afterhyperpolarization to control
57 the pattern of firing (*9*). To determine whether these intrinsic regulators of dopamine neuron action
58 potential firing influence neurotransmitter release dynamics and reinforcement learning, we

59 targeted the inactivation of two potassium channel subunit encoding genes in these cells, Kv4.3
60 (gene name: *Kcnd3*) and BKCa1.1 (gene name: *Kcnma1*) using CRISPR/Cas9 mutagenesis. We
61 found that inactivation of Kv4.3 and BKCa1.1 differentially affected the action potential
62 waveform, neuronal excitability, and patterns of action potential firing. We further show that loss
63 of function (LOF) of these two channels differentially impacted the acquisition, maintenance, and
64 extinction of a reinforced behavioral response that was associated with distinct effects on the
65 patterns of dopamine release during these behaviors. Specifically, loss of BKCa1.1 enhanced burst
66 firing and phasic dopamine release that was associated with enhanced acquisition of instrumental
67 behavior and a heightened extinction burst. In contrast, loss of Kv4.3 increased dopamine neuron
68 excitability, elevated ramping of dopamine release prior to a lever press in a learned instrumental
69 reinforcement task, and facilitated extinction. These findings demonstrate that intrinsic regulators
70 of the action potential waveform influence dopamine release dynamics that control the patterns of
71 behavior during reinforcement learning.

72 **Results**

73 **CRISPR/Cas9 inactivation of Kv4.3 and BKCa1.1**

74 To determine the distribution of *Kcnd3* and *Kcnma1* expression in the VTA, we performed
75 RNAscope *in situ* hybridization to quantify the mRNA levels for these ion channel subunits and
76 the rate-limiting enzyme in dopamine production, *Th*. We find that Kv4.3 and BKCa1.1 subunits
77 are expressed in most dopamine neurons along the rostral-caudal axis (Fig. 1, A-C, fig. S1, A-E),
78 suggesting that these ion channels regulate the activity of the majority of these cells. To selectively
79 inactivate these two channels, we used a viral-mediated, Cre-inducible CRISPR/SaCas9 system
80 (*11*). Adeno-associated viruses (AAV) containing single guide RNA directed to either of the two
81 loci (AAV1-FLEX-SaCas9-sg*Kcnd3* or AAV1-FLEX-SaCas9-sg*Kcnma1*) were generated (Fig. 1,

82 D and H) and injected into the VTA of separate groups of adult *Slc6a3*(DAT)-IRES-Cre mice (fig.
83 S2, A; fig. S3-S4). This yielded high efficiency indel formation and loss of function (LOF) for
84 both channel subunits in dopamine neurons relative to controls (Fig.1, E-G, I-K; fig. S2 B-C; fig.
85 S4, A-C). Specifically, AAV1-FLEX-SaCas9-sg*Kcnd3* nearly abolished the AmmTx-sensitive A-
86 type potassium conductance (Fig. 1, F and G) and AAV1-FLEX-SaCas9-sg*Kcnma1* significantly
87 reduced the iberiotoxin-sensitive BK conductance (Fig. 1, J and K; fig. S4, A-C).

88 **Kv4.3 and BKCa1.1 regulation of dopamine neuron activity**

89 As predicted, Kv4.3 LOF altered the dopamine neuron action potential waveform by
90 increasing the action potential half width and slowing repolarization relative to control and
91 BKCa1.1 LOF cells (Fig. 2, A-D, control mice). BKCa1.1 LOF reduced afterhyperpolarization
92 relative to control and Kv4.3 cells and resulted in a more depolarized resting membrane potential
93 relative to control cells (Fig. 2, A-B, E-F). Neither manipulation affected the peak of the action
94 potential (fig. S4, D). Kv4.3 LOF increased excitability (Fig. 2, G-H) and frequency of
95 spontaneous action potential firing relative to control cells (Fig. 2 I-J). BKCa1.1 LOF did not affect
96 these parameters (Fig. 2 G-J) but shifted the pacemaker-like *ex vivo* activity observed in control
97 and Kv4.3 LOF cells to a more irregular pattern, as evidenced by an increase in the coefficient of
98 variation in the interspike interval (CV-ISI) (Fig. 2, I and K; fig. S4, E).

99 Increased CV-ISI in VTA dopamine neurons of BKCa1.1 LOF mice is consistent with a
100 shift to more burst-like activity (*10*); however, this pattern of firing by dopamine neurons is most
101 accurately assessed by recording neural activity *in vivo*. Using optical isolation (*12*) to identify
102 dopamine neurons during *in vivo* recording (fig. S5, A-C), we found that BKCa1.1 LOF increases
103 the number of spikes in a burst and burst duration of VTA dopamine neurons relative to controls
104 (Fig. 2, L-M), with no effects on baseline firing rate or the frequency of burst events (fig. S5, D-

105 E). Kv4.3 LOF did not alter *in vivo* firing properties under these basal conditions (Fig. 2, L-M; fig.
106 S5, D-E).

107 **Kv4.3 and BKCa1.1 differentially regulate instrumental behavior and dopamine release**

108 To assess whether LOF of these ion channels impacts reinforcement learning, we first
109 trained Kv4.3 and BKCa1.1 LOF mice in an appetitive Pavlovian conditioning paradigm where a
110 lever extension followed by retraction (CS) signals reward (Rew) delivery (fig S6). We did not
111 observe differences between Kv4.3 LOF and BKCa1.1 LOF mice relative to controls (fig. S6, A-
112 C) indicating that dopamine regulation by these channels is not essential for this simple form of
113 learning. Next, mice were transitioned to a fixed-ratio (FR1) schedule of operant reinforcement
114 learning in which lever retraction and reward delivery were contingent on a lever press (LP).
115 Extension of the lever that had previously served as the onset of the CS was now contingent on
116 reward retrieval by making a head entry (HE) into the food hopper (Fig. 3, A); thus, serving as a
117 conditioned reinforcer (CR). BKCa1.1 LOF learned the task faster than control and Kv4.3 LOF
118 mice, as evidenced by a higher frequency of lever presses in the first half of the session on day 1
119 of conditioning in BKCa1.1 mice (Fig. 3, B-C; fig. S6, D-F). Kv4.3 LOF and control mice
120 increased responding between the first and second day that remained elevated on the third day;
121 however, Kv4.3 LOF mice now earned more rewards on the third day of conditioning than
122 BKCa1.1 LOF mice (Fig. 3, B) and had a higher frequency of responding in the first half of the
123 session on day 3 (Fig. 3, D; fig. S6, G-I), a flip of what was observed on day 1 of conditioning.
124 Differences in reinforcement learning were not associated with changes in overall locomotor
125 activity (fig. S7, A-B) and did not reflect changes in motivation (fig. S7, C). These findings
126 indicate that BKCa1.1 LOF enhances acquisition of the learned response, but Kv4.3 LOF enhances
127 performance once the task is learned.

128 To determine how these ion channels contribute to dopamine release dynamics during the
129 distinct phases of reinforcement learning, we expressed the genetically encoded dopamine sensor
130 dLight1.3b (*13*) in the NAc of Kv4.3 LOF, BKCa1.1 LOF, and control mice (fig. S8) and
131 performed fiber photometry during FR1. Mice underwent Pavlovian conditioning followed by the
132 transition to FR1 instrumental conditioning as described above. Due to the restrictive nature of the
133 tethering for fiber photometry recording, mice underwent 5 days of FR1 instead of 3 days and
134 dopamine signals were measured on day 1 and day 5. Control mice showed phasic increases in
135 dopamine time-locked to LP, Rew, and HE/CR on day 1 (Fig. 3, E top row) and day 5 (Fig. 3, E
136 middle row, bottom row). Kv4.3 LOF mice showed highly variable LP and Rew dopamine signals
137 on the first day and HE/CR signals are undetectable (Fig. 3, F top row); clear signals to these
138 events emerged on day 5 (Fig. 3, F middle row, bottom row). BKCa1.1 LOF mice had significantly
139 larger dopamine release events to LP, Rew, and HE/CR on day 1 (Fig. 3, G-H). Dopamine release
140 was decreased on day 5 relative to day 1, except in Kv4.3 LOF mice though the pattern of release
141 is largely consistent (Fig. 3, E-J). Kv4.3 LOF mice displayed a significantly higher peak in the
142 ramp of dopamine release just prior to the lever press on day 5 of conditioning relative to control
143 mice (Fig. 3, K). Cumulative summation (CuSum) analysis of the ramp period revealed that
144 although the ramp was higher in Kv4.3 LOF mice, the start of the ramp, or inflection point, was
145 not different (Fig. 3, L). These data demonstrate that these ion channels regulate the patterns of
146 dopamine release differentially during the acquisition of conditioned response and during
147 performance of the response once it is learned.

148 **Kv4.3 and BKCa1.1 in dopamine neurons differentially influence extinction**

149 Following Pavlovian and FR1 conditioning, a subset of mice underwent extinction training
150 with reward omission (Fig. 4, A). Control mice exhibited an extinction burst in lever pressing on

151 the first day of extinction (higher pressing on day 1 of extinction versus the last day of reinforced
152 FR1 conditioning). This extinction burst is elevated in BKCa1.1 LOF mice relative to control mice
153 (Fig. 4, B; fig. S9, A-C). In contrast, Kv4.3 LOF mice had significantly reduced lever presses
154 during the first day of extinction training relative to both control and BKCa1.1 LOF mice (Fig. 4,
155 B; fig. S9, A-C). The number of lever presses in BKCa1.1 LOF and control mice decreased during
156 the extinction session but remained relatively stable in Kv4.3 LOF mice (Fig. 4, C; fig S9. A-B).
157 All mice performed similarly on the last day of extinction (Fig. 4, B; fig. S9, D-F). Interestingly,
158 lever pressing on the first day of extinction was proportional to the level of responding on the first
159 day of FR1 conditioning (fig. S9, G), suggesting that enhanced acquisition is associated with an
160 invigorated response when the outcome (reward omission) does not match expectation (reward).

161 During extinction training, dopamine signals were largely undetectable to the lever press
162 on the first and last day of extinction training (fig. S9, H-I). In contrast, control mice had a small
163 phasic dopamine response immediately following HE/CR (period A) that quickly returned to
164 baseline (period B) and was further reduced on the fifth day of extinction (Fig. 4, D-F). Kv4.3 LOF
165 mice had a small, but detectable phasic dopamine response during period A that was followed by
166 a sustained signal during period B (Fig. 4, D-F; Wilcoxon sign rank test relative to zero, $P<0.05$).
167 BKCa1.1 LOF mice did not display a phasic increase to the HE/CR period A on either day, but
168 had a significant reduction in the dopamine signal during period B (Fig. 4, D-F), consistent with
169 an enhanced reward prediction error (*14*).

170 **Discussion**

171 Our results confirm that viral-mediated CRISPR/Cas9 mutagenesis is an effective means
172 to study ion channel function in the central nervous system of adult mice. Using this strategy, we
173 showed that Kv4.3 and BKCa1.1 differentially contribute to the action potential waveform and the

174 pattern of action potential firing. We found that altering the firing properties of dopamine neurons
175 to increase pacemaker activity or to induce irregularity and enhance burst firing had differential
176 effects on behavior and dopamine release. These findings support the conclusion that the intrinsic
177 electrophysiological properties of VTA dopamine neurons that contribute to the action potential
178 waveform are critical to the regulation of the neurotransmitter release dynamics that govern
179 behavior.

180 Slowing the action potential repolarization through inactivation of Kv4.3 increased the
181 frequency of action potential firing and excitability. The exact mechanism(s) that underlie Kv4.3
182 regulation of excitability and pacemaker activity are not clear. The most obvious explanation is
183 the contribution of A-type potassium current to the negative feedback during rapid depolarization
184 (*8*). Another potential mechanism is the observation that decreasing Kv4.3 in cardiomyocytes
185 increases sodium conductance (*15*); thus, Kv4.3 LOF may enhance subthreshold sodium currents
186 previously shown to regulate pacemaker activity in VTA dopamine neurons (*16*).

187 Increased ramping of dopamine release prior to lever press observed in Kv4.3 LOF mice
188 following instrumental learning is likely a reflection of an increased activity of VTA dopamine
189 neurons associated with descending GABAergic disinhibitory mechanisms (*17*). Through the
190 regulation of membrane repolarization (*8*) and in the absence of this inhibitory influence, ramping
191 is elevated that enhances performance of the learned response, consistent with previous assertions
192 on the function of ramping (*3-5*). In contrast to previous reports that ramping of dopamine release
193 occurs independently of the activity at the cell body (*5*), our data demonstrate that the intrinsic
194 excitability of the cell, regulated by Kv4.3, contributes to this process.

195 As predicted, BKCa1.1 reduced the action potential afterhyperpolarization (*9*). We did not
196 observe effects of reduced BK current on the action potential width or an increase in pacemaker

197 activity as reported in substantia nigra pars compacta (SNc) dopamine neurons (*9*), suggesting that
198 BK currents regulate firing in distinct ways in these two dopaminergic regions. Consistent with
199 previous reports of decreasing the action potential afterhyperpolarization (*18, 19*), we find that
200 inactivation of BKCa1.1 increased spile irregularity *ex vivo* and burst firing *in vivo*. In the absence
201 of BKCa1.1 channels burst events are prolonged which enhances phasic dopamine release. Based
202 on previous findings (*2, 3, 14, 20, 21*), this elevation in phasic dopamine would be predicted to
203 assign a higher value to action and the outcome to facilitate the acquisition of the instrumental
204 response and enhance the reward prediction error (*2, 3, 14, 20, 21*). In contrast to BKCa1.1 LOF,
205 during early extinction, Kv4.3 LOF promoted a small but sustained activation. We propose that
206 this lack of a prediction error prevents the behavioral extinction burst and facilitates the cessation
207 of responding.

208 We focused on Kv4.3 and BKCa1.1 in dopamine neurons because of their selective and
209 enrichment and their ubiquitous expression across VTA dopamine neurons. It is important to note
210 that these channels are expressed widely throughout the brain where they likely serve similar
211 functions in patterning transmitter release. Further, numerous other ion channels contribute to the
212 action potential. The emergence of many of these ion channels as hotspots of missense mutations
213 in neurodevelopmental disorders (*22*) reinforces the importance of understanding how these
214 channels influence cellular and systems function.

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294
295 **Author contributions:** BJ and LSZ conceived and designed experiments. BJ, MES, ACH, and
296 LZ designed and generated AAVs. BJ and MSK performed surgery. BJ and JEE performed and
297 analyzed in situ hybridization experiments. ACH performed FACS and sequencing. BJ, MAB,
298 AM, NLG, and MAQ performed immunohistochemical validations. BJ and MAB performed
299 immunohistochemical quantification and analysis. BJ and MES performed in vitro
300 electrophysiology. BJ, MK, and YSJ performed in vivo electrophysiology and analysis. BJ, JXY,

AM, and MC performed behaviors. BJ analyzed behaviors. BJ and JXY performed fiber photometry experiments. SNE generated photometry analysis software. BJ and JXY created custom MatLab code for photometry analysis. BJ and LZ further analyzed photometry data. BJ and LSZ wrote the manuscript. All authors provided input and approval of the manuscript.

List of Supplementary Materials

Materials and Methods

Fig S1 – S9

Table S1: Statistics table

Figures and legends

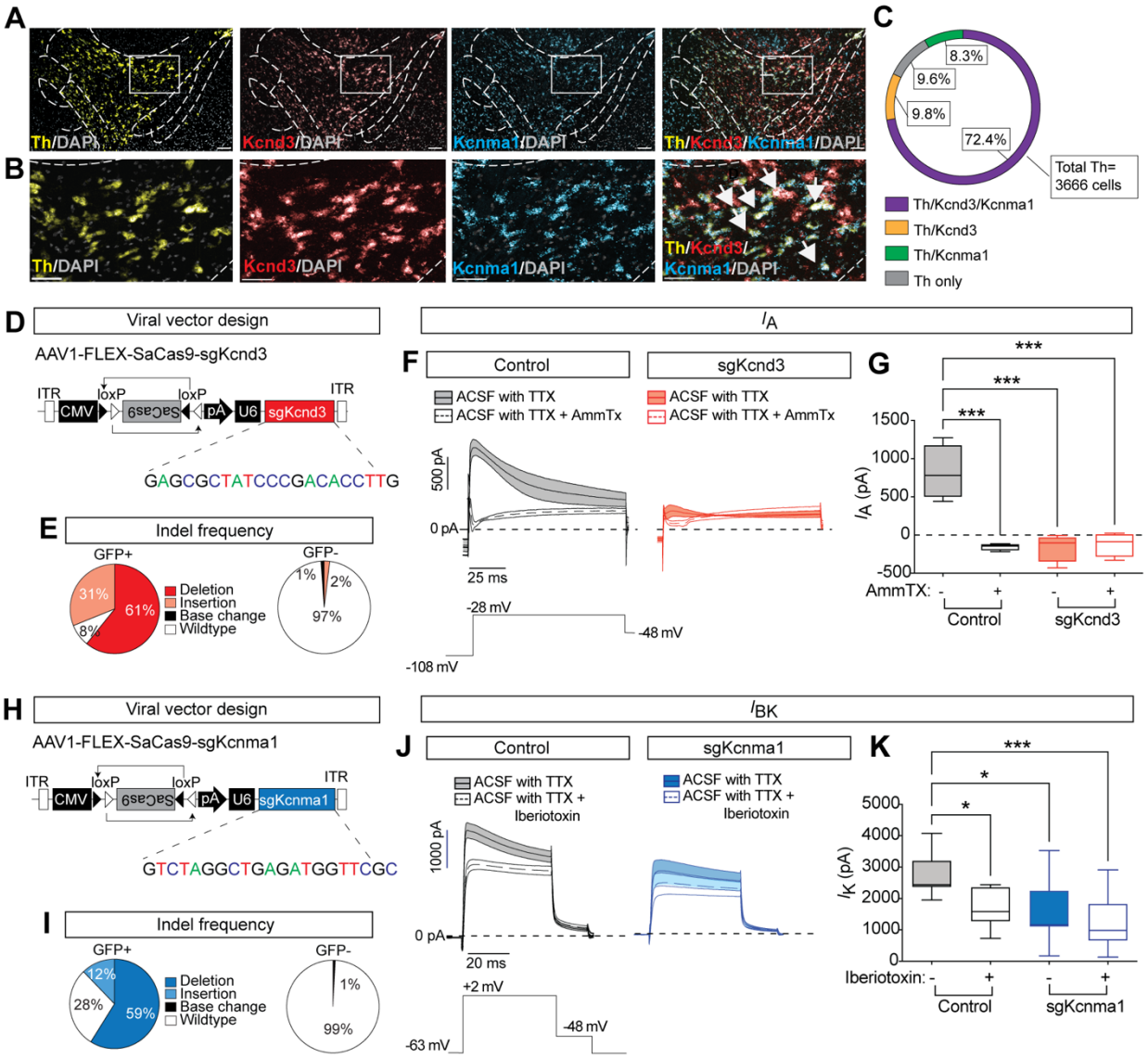

Fig. 1 Targeted mutagenesis of *Kcnd3* and *Kcnma1* in dopamine neurons.

(**A-B**) RNAscope *in situ* hybridization for *Kcnd3*, *Kcnma1*, and *Th*. White arrows: representative *Th/Kcnd3/Kcnma1* overlap. Scale bar, 100 µm (A) and 50 µm (B). (**C**) Quantification of overlap for *Kcnd3, Kcnma1,* and *Th* in the VTA (*N*=3 mice). (**D**) Schematic of AAV1-FLEX-SaCas9-sgKcnd3 targeting virus. (**E**) Proportion of targeted deep sequencing reads with indel mutations following sgKcnd3 targeting (*N*=5 mice). (**F**) Average pre- and post-AmmTx A-Type currents (I_A) from control and sgKcnd3-targeted mice (*N*=4 cells/group) and corresponding depolarization step

to elicit I_A. (**G**) Average peak I_A from (**F**) in control and sgKcnd3-targeted mice (***p<0.001). (**H**) Schematic of AAV1-FLEX-SaCas9-sgKcnma1 targeting virus. (**I**) Proportion of targeted deep sequencing reads with indel mutations following sgKcnma1 targeting (N=5 mice). (**J**) Average pre- and post-iberiotoxin (BK channel blocker) potassium currents (I_K) from control and sgKcnma1-targeted mice ($N_{control}$=11 cells; $N_{sgKcnma1}$=11 cells) and corresponding depolarization step to elicit I_K. (**K**) Peak I_K from (**J**) in control and sgKcnma1-targeted mice following iberiotoxin; notably sgKcnma1 does not have sensitivity to iberiotoxin suggesting functional loss of BK currents (*p<0.05, ***p<0.001).

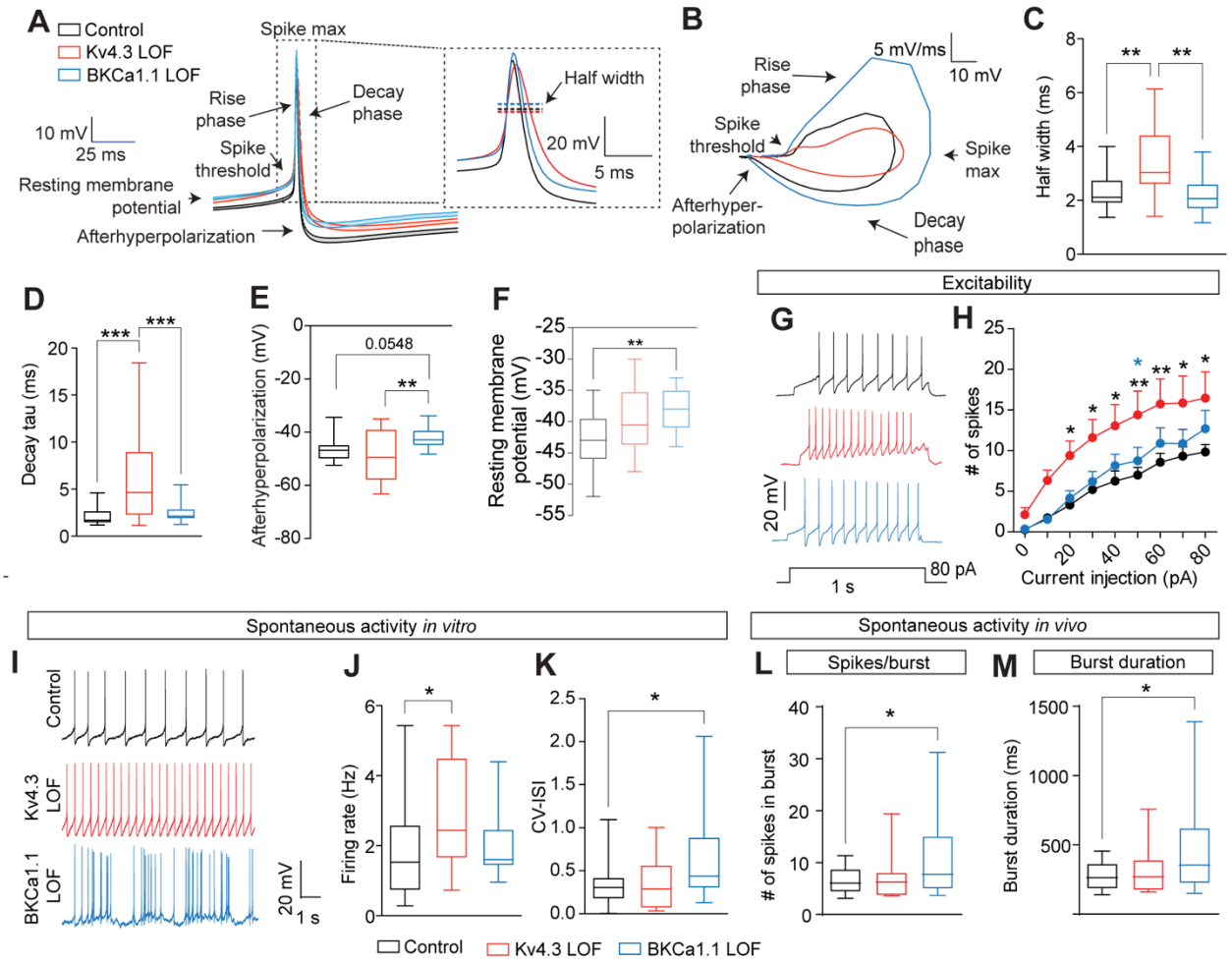

Fig. 2 Kv4.3 and BKCa1.1 LOF alter the dopamine action potential waveform and activity. (**A**) Average action potential waveform from spontaneously active VTA dopamine neurons. (**B**) Representative phase plane plot of action potential dynamics. (**C**) Action potential half width in control, Kv4.3 LOF, and BKCa1.1 LOF cells (**p<0.01). (**D**) Decay kinetics of the action potential (***p<0.001). (**E**) Afterhyperpolarization of the action potential (**p<0.01). (**F**) Resting membrane potential (**p<0.01). (**G**) Representative evoked excitability traces. (**H**) Current-voltage plot (*p<0.05, **p<0.01: black* Kv4.3 LOF vs control, blue * Kv4.3 vs BKCa1.1 LOF; $N_{control}$=19 cells, $N_{Kv4.3\ LOF}$=15 cells, $N_{BKCa1.1LOF}$=20 cells). (**I**) Representative spontaneous activity traces. (**J**) Firing rate of spontaneous activity (*p<0.05). (**K**) CV-ISI of spontaneous activity

(*p<0.05). (**C-F** and **J-K**; $N_{control}$=21 cells, $N_{Kv4.3LOF}$=16 cells, $N_{BKCa1.1}$=19 cells). (**L**) Spikes per burst in VTA dopamine neurons *in vivo* (*p<0.05). (**M**) Burst duration in VTA dopamine neurons *in vivo* (*p<0.05). (**L-M** $N_{control}$=25 cells, $N_{Kv4.3\ LOF}$=13 cells, and $N_{BKCa1.1}$=26 cells).

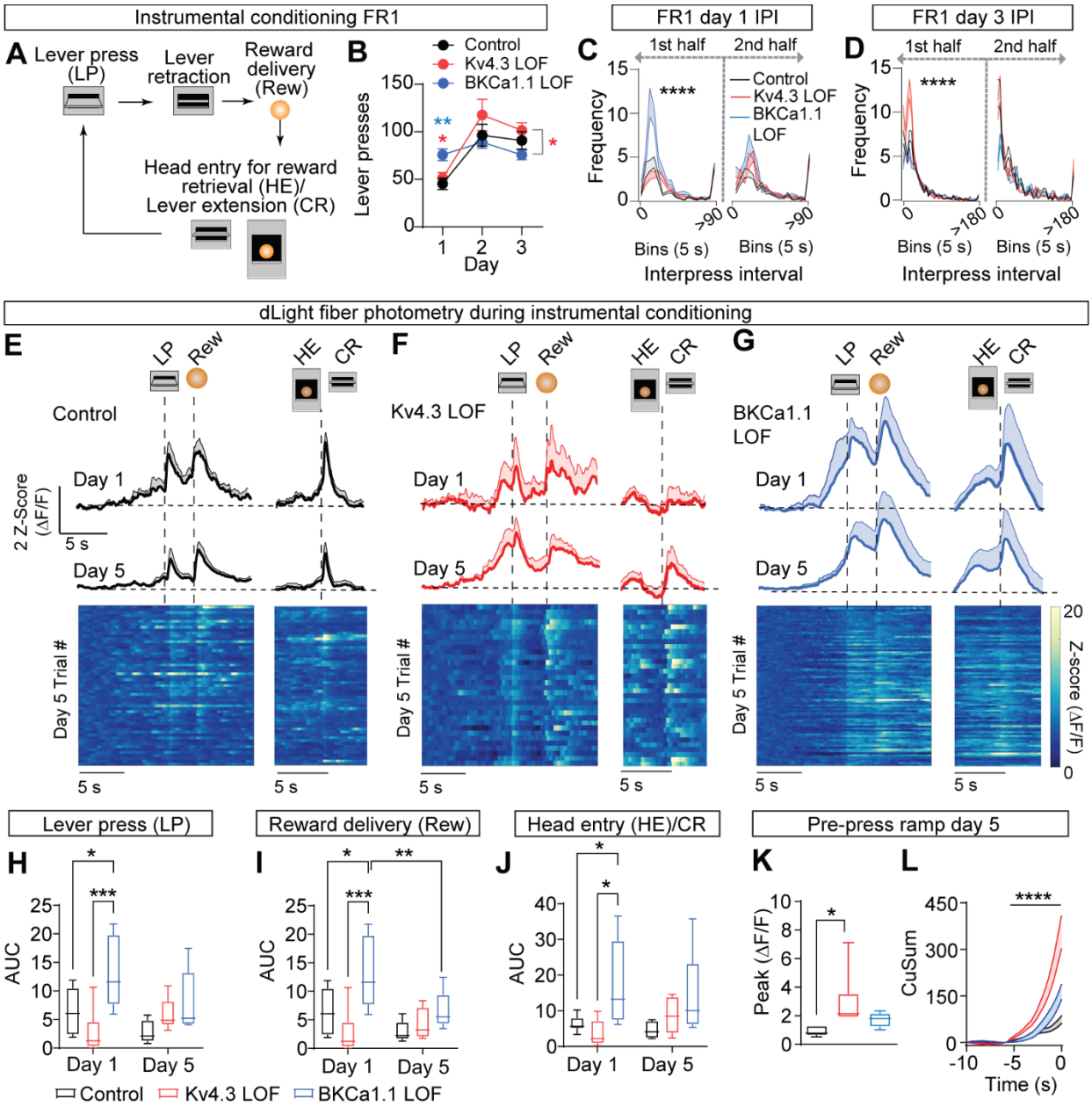

Fig. 3 Instrumental behavior and dopamine signal changes in Kv4.3 and BKCa1.1 LOF mice. (**A**) Schematic of instrumental FR1 conditioning paradigm. (**B**) Lever presses during days 1-3 of FR1 conditioning ($N_{control}$=19, $N_{Kv4.3LOF}$=24, and $N_{BKCa1.1}$=13, **p<0.01, blue=BKCa1.1 LOF vs. control; *p<0.05, red=BKCa1.1 LOF vs. Kv4.3 LOF). (**C-D**) Frequency distribution of lever inter-press intervals during the first and second half of the conditioning session for day 1 (**C**,****p<0.0001) and day 3 (**D**, ****p<0.0001). Average peri-event Z-score for dLight1.3b

signals in the NAc for day 1 (top row) and day 5 (middle row and bottom row) for (**E**) control (*N*=6), (**F**) Kv4.3 LOF (*N*=6), and (**G**) BKCa1.1 LOF (*N*=5) mice. (**H**) Average area under the curve (AUC) for the perievent Z-score dLight1.3b signals post-LP period (3 s) (*p<0.05; **p<0.01; ***p<0.001). (**I**) Average AUC for the Z-score for post-Rew period (5 s) (*p<0.05; ***p<0.001). (**J**) Average AUC for the Z-score for post- HE/CR period (5 s) (*p<0.05). (**K**) Average peak for pre-LP period (*p<0.05). (**L**) Average cumulative summation (CuSum) of the pre-LP period showing similar inflection (start of rise) but significant overall ramping activity in Kv4.3 LOF mice (***p<0.001).

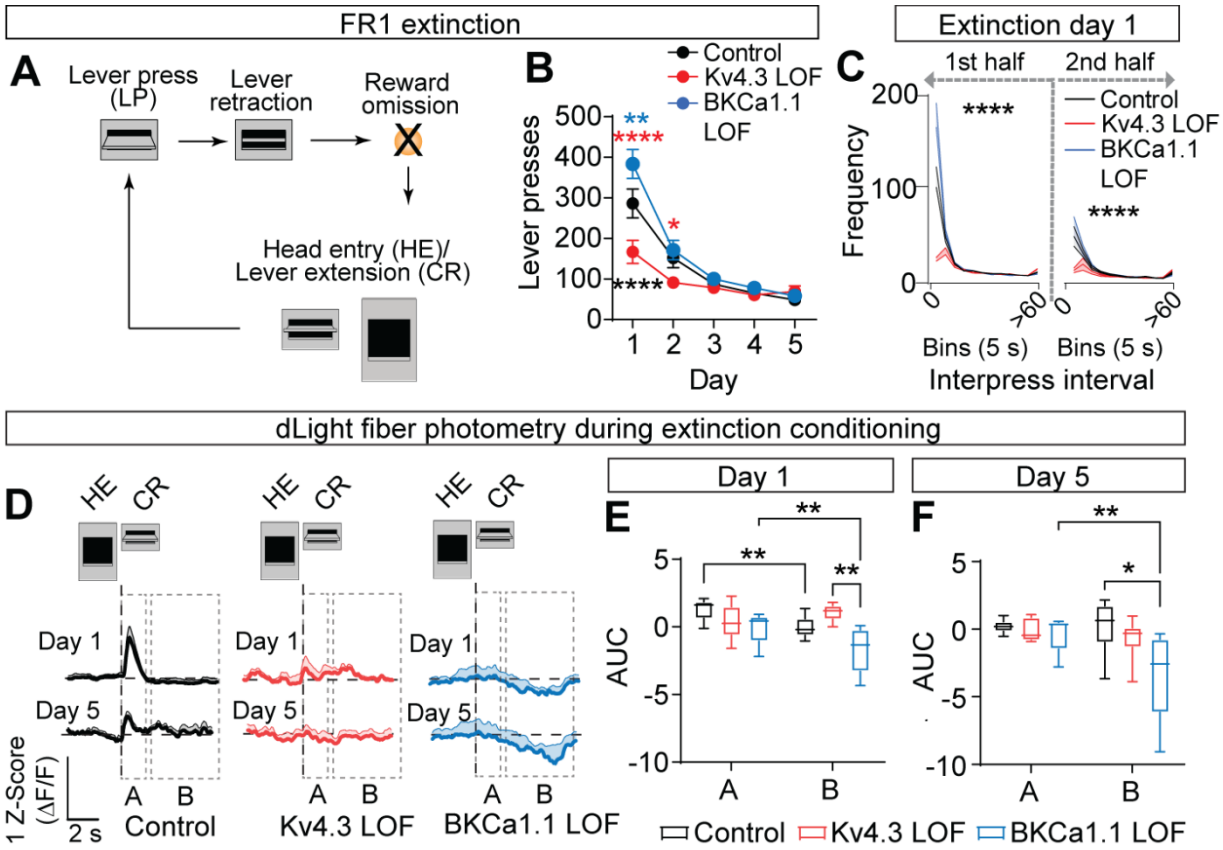

Fig. 4 Extinction behavior and dopamine signal changes in Kv4.3 and BKCa1.1 LOF mice. (**A**) Schematic of instrumental FR1 extinction paradigm. (**B**) Lever presses during days 1-5 of extinction training ($N_{control}$=13, $N_{Kv4.3LOF}$=10, and $N_{BKCa1.1LOF}$=12; **p<0.01, blue= BKCa1.1 LOF vs. control; ****p<0.0001, red=BKCa1.1 LOF vs. Kv4.3 LOF; ****p<0.0001, black= Kv4.3 LOF vs. control). (**C**) Frequency distribution of lever presses during the first and second half of the conditioning session for day 1 of extinction (****P<0.0001). (**D**) Average Z-score for dLight1.3b signals in the NAc during HE/CR in control (*N*=6), KV4.3 LOF (*N*=6), and BKCa1.1 LOF (*N*=5) mice for day 1 and day 5. The signal was separated into the peri-HE/CR period (period A, 2 s) and the subsequent sustained 3 s period during omission (period B) for AUC analysis. (**E**) Average AUC for the Z-score for post-lever HE/CR during the A and B periods on day 1 (**p<0.01). (**E**)

Average AUC for the Z-score ΔF/F for post-lever HE/CR during the A and B periods on day 5 (*$p<0.05$; **$p<0.01$).